DEVELOPMENTS IN HYDROBIOLOGY

Coelenterate Biology 2003

Trends in Research on Cnidaria and Ctenophora

edited by
Daphne G. Fautin, Jane A. Westfall,
Paulyn Cartwright, Marymegan Daly and
Charles R. Wyttenbach

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Trends in Research on Cnidaria and Ctenophora

Proceedings of the 7th International Conference on Coelenterate Biology, held at the University of Kansas, Lawrence, Kansas, U.S.A., 6–11 July 2003

Edited by

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Introduction and acknowledgements

This volume is organized, as was **the Seventh International Conference on Coelenterate Biology**, around six themes. All but one of the contributions on neurobiology were part of a symposium on that subject. Some of the contributions on Reproduction, Development, and Life Cycles were part of the symposium on Axial Patterning. Pioneers in Coelenterate Biology was the subject of a special session. Contributed papers organized around the theme of Ecology included those on Coral Reefs, which constituted the 2003 North American meeting of the International Society for Reef Studies (ISRS). The other two themes were Cnidae, and Taxonomy and Systematics.

Three of the five presentations made in the session on Pioneers in Coelenterate Biology are published here. They provide a wonderful glimpse into the world of our science from the mid-19th century to the mid-20th century. Mary Arai describes the career of her countryman Fraser. Dale Calder's account shows that having an ICCB7 in the middle of North America is not so strange, following as it does in the tradition of Nutting. And George Mackie's vindication of Louis Agassiz's observations provide an ideal segue to the Symposium on the Neuroanatomy and Neurophysiology of Coelenterates.

The symposium has its own introduction, which summarizes the contents of the 15 papers presented in it, and highlights the variability in coelenterates that makes them so interesting and challenging to study. The *sine qua non* of cnidarians, cnidae, continue to provide grist for our research mill. A theme of two of the papers about them in this volume is also variability – its extent in species and individuals. Other techniques applied to sorting out cnidarians and their relationships constitute the section on Taxonomy and Systematics. They range from x-rays to molecules, and show there is no substitute for the time-honored and time-consuming practice of detailed observation. Five of the 13 presentations in the symposium, plus one that one was not given as part of the Axial Patterning symposium, constitute that section. Papers deal with pattern formation both within individuals and in the colonies that many of our marvelously varied animals form. Attention to detail is also evident in the closely-related section on Reproduction, Development, and Life Cycles. All four classes of Cnidaria are represented among those 11 papers; three of them deal with the seldom-studied Cubozoa. Martindale's plenary address and Boero's banquet talk (neither of which is published in this volume) converged on the idea that cnidarians are, deep down, bilateral and triploblastic, a theme that was echoed in some of developmental papers in the volume.

Of the presentations on Coral Reefs that extended through much of a day and a half, eight are published here. As is true of reefs themselves, the theme of variability is very much in evidence. As a sad sign of the times, half of them deal with effects of stress in the animals or detecting stress in the environment. The rest of the papers concerning Ecology run the gamut from freshwater to the deep sea, and include the only plenary address in the volume, Haddock's reflections on research on planktonic coelenterates.

The organizers of ICCB7 were repeatedly challenged about using the term 'coelenterate.' The reason for doing so is two-fold: for continuity of a tradition (see history below) and for inclusiveness, as I have just used it, to cover ctenophores (about which there were several presentations) as well as cnidarians.

Narrative of conference and acknowledgments

ICCB7 was held 6–11 July 2003 at the University of Kansas (KU), Lawrence, Kansas, USA. It began with a reception the evening of Sunday, 6 July, at the University of Kansas Natural History Museum. Sessions were held all day Monday, Tuesday, and Thursday, and the morning of Fri-

day the 11th. The meeting was followed by two days of workshops. In addition to the welcome reception, social events included a Kansas barbeque Tuesday evening and a banquet Thursday evening. Field trips were held on Wednesday.

The 197 registrants presented 139 oral presentations and 67 posters. A plenary talk was given each meeting day. They were, in sequence, 'Glowing gelata: bioluminescence and biodiversity of deep-sea ctenophores and cnidarians' by Steven H. D. Haddock, 'Reefs and organisms change but the play goes on: a billion years of reef evolution' by George D. Stanley, Jr., 'Cnidarians: degenerate triploblastic bilateralians' by Mark Q. Martindale (with co-authors Kevin Pang, Pat Burton, and John R. Finnerty), and 'The symbiotic condition: role of algae in cnidarian hosts' by Gisle Muller-Parker. The banquet talk presented, virtually, by Ferdinando Boero was 'Born to be triploblastic – and bilateral.' The symposium 'Neurobiology,' organized by Jane Westfall, was held Monday and Tuesday, and the symposium 'Axial patterning,' organized by Paulyn Cartwright, was held Thursday. The special session 'Pioneers in Coelenterate Biology' was held Friday morning.

The Organizing Committee of ICCB7 consisted of Daphne G. Fautin (Chair) (KU), Ann F. Budd (University of Iowa), Robert W. Buddemeier (Kansas Geological Survey), Matthew Buechner (KU), Paulyn Cartwright (KU), Marymegan Daly (KU), Paul Enos (KU), Jane Westfall (Kansas State University), and Charles Wyttenbach (KU).

The major financial contributors to ICCB7 were the US National Science Foundation, which provided grants to Daphne Fautin (DEB99-78106 and a supplement to it), Jane Westfall (IBN02-44459), and Paulyn Cartwright (333110), and Allen Press (which is located in Lawrence). Units of KU that contributed financially and in kind to ICCB7 were the Center for Research, Inc., the Department of Ecology and Evolutionary Biology, the Department of Geology, the Department of Molecular Biosciences, the Kansas Geological Survey, KU Bookstores, and the Natural History Museum and Biodiversity Research Center. Other contributors and sponsors were Eastman Kodak Company Scientific Imaging Systems, SpaceSaver/Delta Designs, Hitschfel Instruments, Fisher Scientific, VWR International, Nalge Nunc International, ISRS, Free State Brewery (Lawrence), Ray's Liquor, HyVee, Office Depot, and H. Stevan Logsdon, wildlife artist.

Special thanks are due to people who provided logistical help. Registration, arrangements for space and transportation, etc. was handled by Sharon Brookshire, assisted by Ellen Stauffer and Rebeccas Frakes of the Division of Continuing Education, Kansas State University. Coordinating facilities at the Kansas Union was Lisa Kring. Audio-visual services were by Ganesh Shankaran, assisted by Asif Iqbal, John Guinotte, and Kai Raab. Transportation and barbeque arrangements were by Lorraine Murtha. The barbeque was at Pendleton's Country Market and Farm, the chief cook was Joe Markley, chickens were grown at the Clark Family Farms, bison was raised by Lone Star Lake Bison Ranch and Meat Company, pies and rolls were made by MMMS Baked Goods (Mary, Margaret, Molly, and Sarah), and beer was from the Free State Brewery.

Proceedings

In addition to the contribution of registration fees to the production of this volume, US NSF grant IBN02-44459 to Jane Westfall helped pay for publication of papers that were part of the symposium 'Neuroanatomy and Neurophysiology of Coelenterates.' Ganesh Shankaran as well as students employed through Sloan Foundation funds awarded through the Census of Marine Life helped with the logistics of compiling the volume manuscripts.

Previous ICCBs and resulting volumes

3–4 March 1965 – The Zoological Society of London, UK

Organizing Committee: W. J. Rees (chair), Sir Fredrick Russell, C. F. A. Pantin, Cadet Hand, D. M. Ross.

Number of Attendees: 27

Proceedings: Rees, W. J. (ed.), 1966. *The Cnidaria and their Evolution*. Symposia of the Zoological Society of London 16. London: Academic Press. 449 pages.

16–19 October 1972 – Shirahama and Kushimoto, Japan

Organizing Committee: Tohru Uchida (chair), A. Brinckmann-Voss, G. O. Mackie, W. A. Müller, D. M. Chapman, S. Kawaguti, H. R. Randall, C. Edwards, H. Thiel, L. M. Passano, M. Tamasige, R. Weill, M. N. Arai, E. A. Robson, E. Harada, M. Yoshida, D. Ross

Number of Attendees: 46

Proceedings: Tokioka, Takasi (ed.), 1973. *Recent Trends in Research in Coelenterate Biology*. Publications of the Seto Maine Biological Laboratory 20. 793 pages.

10–13 May 1976 – University of Victoria, Victoria, BC, Canada

Organizing Committee: George O. Mackie (chair), Mary Needler Arai, Donald M. Ross

Number of Attendees: 139

Proceedings: Mackie, G. O. (ed.), 1976. *Coelenterate Ecology and Behavior*. New York: Plenum Press. 744 pages.

4–8 September 1979 – Interlaken, Switzerland

Organizing Committee: Pierre Tardent, Ruth Tardent (co-chairs), staff members and students of the Zoological Institute of the University of Zürich

Number of Attendees: 109

Proceedings: Tardent, P. & R. Tardent (eds), 1980. *Developmental and Cellular Biology of Coelenterates*. Amsterdam: Elsevier/North Holland Biomedical Press. 499 pages.

10–14 July 1989 – University of Southampton, UK

Organizing Committee: R. G. Hughes (chair), P. F. S. Cornelius, E. A. Robson, M. A. Carter, J. A. E. B. Hubbard, R. B. Williams

Number of Attendees: 250

Proceedings: Williams, R. B., P. F. S. Cornelius, R. G. Hughes & E. A. Robson (eds), 1991. *Coelenterate Biology: Recent Research on Cnidaria and Ctenophora*. Boston: Kluwer Academic Publishers. 742 pages.

16–21 July 1995 – The Leeuwenhorst Conference Center, Noordwijkerhout, The Netherlands

Organizing Committee: S. van der Spoel (chair), R. M. L. Ates, J. Bleeker, J. C. den Hartog, J. H. M. Kouwenberg, A. C. Pierrot-Bults, P. H. Schalk, R. W. M. van Soest, H. W. van der Veer, J. Vermeulen

Number of Attendees: 130

Proceedings: den Hartog, J. C., L. P. van Ofwegen & S. van der Spoel (eds), 1997. *Proceedings of the 6th International Conference on Coelenterate Biology*. Leiden, The Netherlands: Nationaal Naturhistorisch Museum. 542 pages.

Part I
Pioneers in Coelenterate Biology

Hydrobiologia **530/531:** 3–11, 2004.
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Charles McLean Fraser (1872–1946) – his contributions to hydroid research and to the development of fisheries biology and academia in British Columbia

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Key Words: Canada, fisheries, Fraser, history, hydrozoa, Pacific ocean

Abstract

Charles McLean Fraser (1872–1946) is best remembered for his work on hydroids, but he was also involved in a much broader range of activities during the development of marine biology on the Pacific coast of Canada in the first half of the 20th century. He first became interested in hydroids when, as a graduate student at the University of Toronto, he collected from the floating laboratory near Canso, Nova Scotia. In 1903 he began teaching at the high school in Nelson, British Columbia, and collected hydroids at various locations on the Pacific coast. He completed a Ph.D. under the direction of C. C. Nutting at the University of Iowa. Fraser had worked at the Pacific Biological Station in 1908, the first year it was open, and in 1912 he became its second curator. In the latter capacity he was a member of a commission on sea lions, and worked on such commercially important species as salmon and herring. From 1920 to 1940 he was Head of the Department of Zoology at the University of British Columbia and continued marine work there. He was an influential member of societies such as the Royal Society of Canada and the Pacific Science Association. In the decade before the Second World War he also participated in the Allan Hancock Pacific Expeditions which collected from Southern California to Peru and east into the Caribbean Sea. His hydroid work culminated in the publication of three books on hydroids of the Pacific and Atlantic coasts of North America and their distribution and relationships.

Introduction

The most lasting legacy of Charles McLean Fraser (Fig. 1) is his work on the taxonomy and distribution of hydroids, a subject he pursued throughout his professional career. In the last 10 year of his life it culminated in the publication of three monographs on American hydroids (Fraser, 1937a, 1944, 1946) as well as two volumes on the Allan Hancock Expeditions to the eastern Pacific. To his contemporaries, however, Fraser was better known as one of the developers of fisheries biology and oceanography on the Pacific coast of Canada. As Curator and Director of the Pacific Biological Station, 1912–1924, and as Professor of Zoology at the University of British Columbia, 1920–1940, he played a preeminent role in that development.

Fraser died December 26, 1946, at his home at 4585 West Sixth Avenue, Vancouver. Formal obituaries written by Clemens (1947, 1948) and Foerster (1948), and an appreciation and list of most of his publications published by Schmitt (1948), previously provided some details about his career. The present paper emphasizes his achievements in the context of the localities and period in which he was working.

Figure 1. Charles McLean Fraser. Courtesy of Fisheries and Oceans Canada.

Background and training

Charles McLean Fraser was born June 1, 1872, in Bluevale, Southern Ontario, a village northwest of Kitchener in Huron County (Cattell & Cattell, 1927). He attended the Ontario College of Education and taught public school before entering the University of Toronto (Clemens, 1947). At the university he obtained a B.A. with honours in 1898. Sources indicate that he also received an M.A. from the Department of Biology under Professor Ramsay Wright in 1903 (Cattell & Cattell, 1927; Clemens, 1947; Foerster, 1948) or 1904 (Craigie, 1966, p. 39, 68), one of the first to be granted by the department. It has not been possible to verify the correct date in the present university library or archives (D.R. Calder pers. commun.).

During the 19th century, preliminary exploration of the Canadian coelenterate fauna was done primarily by Canadian geologists or by foreign scientists (Arai, 1992). Canada's first Marine Biological Laboratory was a one-storey building on a scow. Supported by the Government of Canada and operated by a Board of Management, it was moved to various locations on the east coast in the summers of 1899–1906 (Rigby & Huntsman, 1958; Johnstone, 1977). Most of the workers at the laboratory were volunteers from Canadian universities.

During the summers of 1901 and 1902, Fraser accompanied R. Ramsay Wright, then assistant director of the laboratory, to Canso, northeast Nova Scotia, which was then the largest fishing center on the east coast (Rigby & Huntsman, 1958). In both summers he collected and prepared specimens for the University of Toronto Museum of Zoology. In 1902, he also made his own first collection of hydroids by examining the wharves of the harbour, by dredging in Chedabucto Bay, and by trawling offshore banks with fishermen (Fraser,

1913b). As described in a letter from Fraser (quoted by Rigby & Huntsman, 1958), the dredging in a small gasoline launch in rough seas was very dangerous.

The first marine station in British Columbia was the Minnesota Seaside Station established from 1901 to 1907 by a botanist, J. Tilden, of the University of Minnesota (Johnstone, 1977). It was in a very isolated location at Botany Bay, near Port Renfrew, on the southwest coast of Vancouver Island. Fraser spent some time there shore-collecting in the summer of 1903 (Fraser, 1911). During the fall of the same year, Fraser moved inland to Nelson, British Columbia, where he became science master in the high school and, in 1904, the principal.

The Board of Management of the Marine Biological Stations established the Pacific Biological Station at Departure Bay, near Nanaimo, on the east coast of Vancouver Island. It was erected during the fall and winter of 1907–1908 under the supervision of the first curator, the Reverend G. W. Taylor, and was ready for operation in the spring of 1908 (Rigby & Huntsman, 1958; Johnstone, 1977). Fraser spent the summers of 1908 and 1909 shore collecting and dredging for hydroids based at this station (Fraser, 1911).

In September 1910 Fraser moved to the University of Iowa in order to complete a Ph. D. under C. C. Nutting (Fraser, 1911) . At that time graduate training in zoology was not yet available in Canada at the Ph.D. level (Dymond, 1949) , although it had already been available in the United States since the latter part of the previous century (Benson, 1988). Nutting was the logical choice as a supervisor for work on collections from the west coast of America since he had described the hydroids collected by the Harriman Alaska Expedition (Nutting, 1901a) and was engaged in writing a multivolume monograph on American hydroids (see Calder (2004) for further information on Nutting).

From the west coast of Canada, Fraser brought with him his own hydroid collections from the waters around Vancouver Island, as well as some from as far north as the Queen Charlotte Islands, made by other workers at the Pacific Biological Station. Nutting also placed at his disposal two collections made in the San Juan Archipelago. Based on these collections and previous literature, Fraser published both an extensive paper on the hydroids of the west coast of North America (Fraser, 1911) and a list of the hydroids of Vancouver Island (Fraser, 1913a).

Fraser was awarded a fellowship at the University of Iowa, 1911–1912 (Cattell & Cattell, 1927). During the summer of 1911 he collected on the east coast of the United States at South Harpswell, Maine; Woods Hole, Massachusetts; and Beaufort, North Carolina (Fraser, 1912a, b). At Beaufort he collected 51 species and was able to produce an extensive key to the fauna of the area, which provided good training in the classification of hydroids. Nutting allowed him to include the family Hebellidae from an unpublished manuscript. Fraser also benefited from Nutting's previous work at Woods Hole (Nutting, 1901b), and from his extensive collections and library. All in all, the 2 years with Nutting provided Fraser with a solid training in hydroid taxonomy.

While at Iowa, Fraser also worked on a paper on the hydroids of Nova Scotia, based on his own earlier collections near Canso and on a collection amassed by John Macoun from an area near Barrington Passage at the south of the province. Fraser published the study soon after his return to Canada (Fraser, 1913b).

The drawings for these first papers were done by Fraser's wife, Clara Cassidy Fraser. She assisted him in this way until after completion of his book on *Hydroids of the Pacific Coast of Canada and the United States* (Fraser, 1937a). Later publications were illustrated by U. Dale, J. Hart, or M. McCrea. There is no indication that Fraser ever drew his own illustrations beyond very preliminary sketches.

Pacific Biological Station

Fraser returned to the Pacific Biological Station April 1, 1912 (Rigby & Huntsman, 1958). The Reverend Taylor, who had become seriously ill in 1910, was provided with assistance although retained as official Curator (Rigby & Huntsman, 1958; Johnstone, 1977). For the summer of 1912, J. P. McMurrich was appointed Director and Fraser was engaged to fulfill most of the curatorial duties. On October 1, 1912, shortly after Taylor's death in August, Fraser was appointed as year-round Curator. The position involved his own scientific work, arranging scientific facilities for the

seasonal volunteers, and overseeing the unpretentious residential facilities, in which he was assisted by his wife. Following his appointment in 1920 as Professor at the University of British Columbia, Fraser became Director of the station, spending the summer months there and making regular visits at other times of the year. Since this part-time arrangement was not very satisfactory, W. A. Clemens was appointed as a year-round Director in the spring of 1924.

In 1912 an Act of Parliament replaced the Board of Management by the Biological Board of Canada (Johnstone, 1977; Needler, 1984). The act gave the Board greater financial independence and authority to determine its own policies. Although the work on fauna and flora by volunteers from the universities continued, the Board broadened the scope of its activities and related them more directly to the practical problems of the fisheries. This exerted particular pressure on Fraser, because as the only full-time scientist at the Pacific Biological Station, he had to conduct fisheries related research (Fraser, 1915). In the summer of 1912 he initiated work on herring, and over the following years initiated and carried out projects on a wide variety of fisheries-related matters.

Many of the fisheries topics that Fraser investigated continue to be important today. Others, such as a search for lobster planting areas, proved unsuccessful for both Fraser and following investigators (Fraser, 1915, 1916). The most important fishery was for the five species of salmon. Fraser conducted research on life histories, growth rates, scales, grading, rearing, parasitic copepods, migration, predation, and food of salmon, and wrote several papers (Schmitt, 1948; Rigby & Huntsman, 1958; Johnstone, 1977). He completed the work on the life history of herring (Fraser, 1922) and surveyed the damage from marine wood borers to British Columbia wharves (Fraser, 1923, 1925). In 1914, with A. T. Cameron, he also initiated a series of oceanographic studies, including regular measurements of temperature and salinity and studies of their effects on the distribution of fauna and flora (Rigby & Huntsman, 1958).

In 1915 the Dominion Government offered a controversial bounty to reduce the number of sea lions. Fraser became a member of a three-man commission appointed by the Biological Board that investigated the effect of the bounty, 1915–1917 (Newcombe et al., 1918). They visited several rookeries along the British Columbia coast, but obtained only an unsatisfactory amount of data on feeding and hence on damage to fisheries (Rigby & Huntsman, 1958). The killing of sea lions continued for many decades.

Although his research during this period was largely on fisheries subjects, Fraser did continue to work on hydroids, both by his own collecting and by examining collections sent to him by others. Collections made by Fraser and by J. P. McMurrich in British Columbia, as well as data from a 2-week trip Fraser made to Friday Harbor in Washington State to collect and examine university collections, yielded enough new information to justify another paper on hydroids of the region by 1914 (Fraser, 1914). Fraser also received extensive collections from the Atlantic Biological Station on the east coast of Canada. These enabled him to publish a paper in 1918 on the distribution of hydroids of eastern Canada (Fraser, 1918), followed by a key (Fraser, 1921) which was reprinted in the Canadian Atlantic Fauna series.

Fraser's duties included transmission of information on marine matters to local and international audiences. As indicated by reports in the local newspapers, he was a popular speaker for groups such as the Natural History Society, Victoria, the Vancouver Institute, and the British Columbia Academy of Science. He was a charter member of the Academy and served as its President from 1916 to 1918 (Foerster, 1948). In addition he was elected a fellow of the Royal Society of Canada in 1916 and published several papers in its transactions (Clemens, 1947).

The Pacific Fisheries Society, based primarily in the United States, was founded in 1914 (Anonymous, 1914). At the first meeting, on the University of Washington campus, Fraser gave a talk about the Pacific Biological Station (Fraser, 1915). He also spoke at the next meeting in San Francisco, presenting a talk on the growth of chinook salmon. Obviously esteemed, he served as the Society's Vice-President in 1917–1918, and as its President in 1919 (Foerster, 1948).

In 1920, the first Pan-Pacific Science Congress was held in Honolulu for 3 weeks to present papers on general topics and formulate programs for future cooperative scientific work in the Pacific region (de Vries, 1930). Fraser, who attended as

the representative of the Biological Board, was the only Canadian among the 99 scientists who attended. He made presentations on fisheries and oceanography, chaired discussions, and was appointed to a small committee charged with making arrangements for subsequent triennial congresses (de Vries, 1930; Schmitt, 1948; Rigby & Huntsman, 1958). In 1923, again as the representative of the Biological Board, he attended the second Pan-Pacific Science Congress held in Melbourne and Sidney, Australia.

University of British Columbia

In 1915 the independent University of British Columbia replaced McGill University College of British Columbia as the provincial university (Logan, 1958). During the period between the two world wars, the university developed from a small college in central Vancouver to a major university with an extended campus at Point Grey west of the main city. The move from the city facilities, very overcrowded due to the post-war bulge of students, to the Point Grey campus took place in 1925.

In the early months of 1919 and of 1920 Fraser obtained leave from the Pacific Biological Station to lecture at the university (Rigby & Huntsman, 1958). In 1920 he was appointed Professor and Head of the Department of Zoology (Logan, 1958). Fraser retired in 1940 and he was appointed as Emeritus Professor in 1941. A year later the university awarded him the L.L.D. (*honoris causa*) (Cattell, 1944).

Fraser was a popular undergraduate professor. He was an excellent lecturer who taught both the introductory course in zoology and advanced courses. I. McTaggart Cowan, an undergraduate student and later a member of the university faculty, remembers his ability to give well-organized and detailed lectures without resorting to notes (I. M. Cowan, pers. commun.). In the laboratory Fraser provided the necessary facilities and equipment and then allowed students to work as independently as possible. He was one of the founders of he University of British Columbia Biological Discussion Club (Foerster, 1948). Outside of the classroom, Fraser was interested in student athletics, especially basketball and rugby. At his retirement the undergraduate students awarded him the honorary gold Big Block award (Anonymous, 1940).

Fraser continued to be concerned with the welfare of his students after they graduated. McTaggart Cowan completed a Ph.D. at the University of California at Berkeley, but in 1935 was having difficulty finding a job in the depths of the depression. Fraser advised him that there was a vacancy at the Provincial Museum and that he should apply for a job which he subsequently received (Corley-Smith, 1989). Some students were attracted by Fraser to fisheries work. For example, R. E. Foerster credited Fraser with diverting him from medicine to marine biology (Johnstone, 1977, p. 112). Foerster became Fraser's masters student, examining live hydromedusae he collected himself in 1921 at the Pacific Biological Station and preserved specimens collected 1912–1921 by previous workers at the station (Foerster, 1923; Johnstone, 1977). He went on to become a well-recognized expert on sockeye salmon and a director of the Biological Station (Ricker, 1979).

After leaving the Pacific Biological Station in 1924, Fraser's own fisheries research decreased, although at the request of the Biological Board he and G. M. Smith made a series of studies on the commercial clams. By 1931 he had authored or coauthored six papers on the ecology and life history of clams (Schmitt, 1948). He remained active on advisory bodies. W. A. Clemens, the next station director, made him a member of a Fisheries Advisory Committee, which recommended research projects to the Biological Board (Johnstone, 1977). Both Fraser and Clemens were appointed members of the Advisory Council of the International Halibut Commission (Rigby & Huntsman, 1958). Later Fraser was a member of the Associate Committee on Oceanography of the National Research Council from at least 1928 to 1937 (Schmitt, 1948).

During his time at the university, Fraser concentrated his research on hydroids. He traveled widely and continued his hydroid collecting whenever it was possible. In the summer of 1935 he collected from the Canadian Hydrographic Service survey vessel, the *Wm. J. Stewart*, off the Queen Charlotte Islands. The size of the ship allowed collection off the little known west coast of the islands (Fraser, 1936a, b).

Fraser also received collections sent from a variety of sources. Several of these expanded the knowledge of distributions of species around North America, especially extensive collections from the United States National Museum and the Museum of Comparative Zoology at Harvard College. He received two batches of hydroids collected in Japan by His Majesty, the Emperor of Japan (Fraser, 1935, 1936c). These collections excited the interest of the local press when Fraser completed the papers for the Emperor and received two beautiful cloisonne vases as a gift.

In 1941–1942 Fraser was President of Section V of the Royal Society of Canada. In his presidential address he traced the history of marine zoology in the Northeast Pacific (Fraser, 1942). He was also active in the Oceanographic Society of the Pacific, serving as Vice-President in 1935 and President in 1936 (Foerster, 1948).

Fraser and his wife attended the Pacific Science Congresses in Tokyo (1926), Batavia-Bandoeng, Java (1929), Vancouver and Victoria, British Columbia (1933), and Berkeley and Stanford (1939). In the Third Pan-Pacific Congress in Tokyo Fraser represented the University of British Columbia, the Canadian National Research Council, and the Royal Society of Canada (Proceedings of the Third Pan-Pacific Science Congress, 1928). He advocated international cooperation in the investigation of pelagic fish eggs and larvae and for protection of useful aquatic fauna, and he presented a paper on marine borers. The Fourth Pacific Science Congress in Java was the first held under a permanent Pacific Science Association and its administrative arm, the Pacific Science Council (Proceedings of the Fourth Pacific Science Congress, 1930). Fraser was a member of the Pacific Science Council and represented the Government of Canada and the National Research Council. He returned from this meeting via a number of scientific institutions in southern Asia and Europe (Clemens, 1947).

Fraser played a particularly active part in the Fifth Pacific Science Congress which was hosted by the National Research Council of Canada in Vancouver and Victoria June 1–14, 1933 (Proceedings of the Fifth Pacific Science Congress, 1934). He was a member of the Executive Committee for the meeting and he chaired the Division of Biological Sciences and the Section on Oceanography. The congresses had increased steadily in size, and the Canadian meeting included more than 300 members and other participants. In addition, more than 100 women accompanied their husbands and were officially entertained.

Allan Hancock expeditions

During the last 14 year of his life Fraser was deeply involved with the Allan Hancock Pacific Expeditions, which collected in the eastern tropical and subtropical Pacific Ocean and the Caribbean Sea. Allan Hancock was Director of the Hancock Foundation for Scientific Research of The University of Southern California, Los Angeles. A rich man, he was able to develop a series of ships for scientific exploration. The *Velero III* was a luxurious motor cruiser well-equipped for sampling and maintaining fauna for the work of up to a dozen scientists (Fraser, 1943a). Between 1932 and its requisition by the United States navy in 1941 the *Velero III* sailed on 10 expeditions over various routes extending as far south in the Pacific as Peru and also into the southern Caribbean Sea, as far east as Trinidad and Tobago. Fraser participated in the expedition December 30, 1933, to March 14, 1934, which sailed from Los Angeles to the Galapagos Islands and then north along the coast from Ecuador to Mexico. In 1940–1941, immediately after retirement from the University of British Columbia, he served as a Research Professor at the University of Southern California (Cattell, 1944). During this time he participated in the short Tenth Expedition (February 22, 1941 to March 2, 1941) which sailed down the west coast of Baja California to Isla Cedros.

In addition to his own field collecting, Fraser received hydroid specimens sorted from the collections of other expeditions. The collections were very extensive, and he noted that even the collections from the relatively short trips in this area after 1938 required 2250 separate identifications (Fraser, 1948). He wrote five papers on the hydroids from the Pacific and one on the Atlantic (including two published posthumously). He described 144 new species from the tropical and subtropical Pacific and six from the Caribbean (Schmitt, 1948). Aside from his hydroid work, Fraser also wrote the general accounts of the

scientific work done from the *Velero III* in the Pacific between 1931 and 1941 (Fraser, 1943a). The descriptions of the ship, the personnel, the geographical and biological associations, and collecting stations comprised the first volume of the Allan Hancock Pacific Expeditions records.

Hydrozoan legacy

Fraser compiled his extensive work on the taxonomy and distribution of North American hydroids into three monographs: one each on the hydroids of the Pacific and Atlantic coasts and one on the distribution and relationships of those hydroids (Fraser, 1937a, 1944, 1946). This was a monumental undertaking which still dominates the field in North America over 50 year later.

In his career Fraser described more than 230 new species, but he unfortunately designated holotypes for only 33 species in the four papers pertaining to collections from the United States National Museum (Fraser, 1937b, 1940, 1941, 1945). A few Atlantic specimens described in Fraser (1943b) were deposited in the Museum of Comparative Zoology, Harvard University. Much of the material from the Allan Hancock expeditions was returned by Fraser to the Allan Hancock Foundation and is presently in the Santa Barbara Museum of Natural History. The rest of his material remained in Fraser's private collections at the University of British Columbia at his death or had been discarded. Thirty years later much of it was in very poor condition from lack of curation while it was in university storage, and I was given it to sort and deposit in the British Columbia Provincial Museum. I published a list of that portion of the collection that pertained to any of the new species he had described (Arai, 1977). Only 144 of his species were found and only a few specimens of those were labeled 'type' or 'co-type'. Specimens in both the Santa Barbara and British Columbia collections need to be examined and lectotypes designated as necessary.

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From birds to hydroids: Charles Cleveland Nutting (1858–1927) of the University of Iowa, USA

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Abstract

Charles Cleveland Nutting (1858–1927) began his academic career at the University of Iowa, and at the Natural History Museum of the university, in 1886. A naturalist with a bent for ornithology, he became interested in cnidarians, and especially hydroids and alcyonarians, as an outcome of expeditions to the Bahamas in 1888 and 1893. Most noteworthy among his works on Cnidaria were three classic volumes on American hydroids, dealing respectively with Plumulariidae (1900), Sertulariidae (1904), and Campanulariidae and Bonneviellidae (1915). Other papers dealt with hydroids from Britain, Alaska and Puget Sound, Woods Hole and vicinity, Hawaii, and the Philippine region. He founded two new families, seven new genera, and 175 new species of Hydrozoa, ranking him among the top three authors of species names in the group. Nutting also described 206 new nominal species of Alcyonaria. Important among his works on alcyonarians were monographs on Indonesian material from the Siboga expedition, and on 'Albatross' collections from Hawaii, Japan, and elsewhere in the northwest Pacific. While birds and later hydroids were Nutting's primary research interests, much of his 41-year professional career was devoted to academic duties at the University of Iowa and to advancement of its Natural History Museum. As museum Director, he significantly expanded the collections, organized exhibits and public programs, and petitioned for satisfactory facilities and funding. Under his leadership, in whole or in part, expeditions to collect specimens for research, teaching, and exhibition were carried out to the Bay of Fundy (1890), Manitoba (1891), the Bahamas and vicinity (1893), Barbados and Antigua (1918), and Fiji and New Zealand (1922). In tribute to Nutting and his contributions to biology, one genus and more than 30 species are named in his honor.

Introduction

Research was initiated on Hydrozoa of North American waters by 19th century naturalists such as William Stimpson, J.W. Dawson, Joseph Leidy, John McCrady, Louis and Alexander Agassiz (see Mackie (2004) for some of the former's work), A.S. Packard, A.E. Verrill, G.J. Allman, S.F. Clarke, W.K. Brooks, and J.W. Fewkes. These pioneers were followed, at the turn of the 20th century, by another generation of hydrozoan taxonomists. Among those of particular distinction during that period were C.C. Nutting, a student of hydroids, A.G. Mayer, a specialist on hydromedusae, and C.W. Hargitt, who worked on both hydroids and medusae. Knowledge of Hydrozoa worldwide was advanced significantly by their investigations.

A brief biography of C.C. Nutting, and an account of his contributions on hydroids, is the purpose of this paper. Nutting published

important works on other groups as well, but his most noteworthy scientific accomplishments were made in hydrozoan biology.

The early years

Charles Cleveland Nutting was born on 25 May 1858 in Jacksonville, Illinois, U.S.A., a son of the Rev. Rufus Nutting and Margaretta Leib Hunt Nutting. His father was a Presbyterian minister (earlier a Congregationalist) and a professor of Greek at Illinois College in Jacksonville. Rufus later moved to Blackburn College in Carlinville, IL. Charles had two brothers, one of whom died in infancy, and four sisters. 'Charlie' reportedly had 'the knack for experimenting himself into trouble' and 'the merriest of blue eyes and the most infectious laugh and the keenest sense of humor' (Taylor, 1937, 1943). After early schooling in Jacksonville, Charles attended Shortridge High School in Indianapolis, Indiana. Among teachers who influenced him there was David Starr Jordan, later a famous ichthyologist and president of Stanford University. A keen naturalist at a young age, Nutting entered Blackburn College in 1876, receiving an A.B. in 1880. Following graduation he worked for more than a year as a paymaster and surveyor in Colorado, at the time a rough-and-ready frontier region, before receiving an A.M. from Blackburn in 1882. He then undertook ornithological collecting expeditions for the Smithsonian Institution in Costa Rica (1882), Nicaragua (1883), and Florida and the West Indies (1884–1985), and he maintained close ties with the Smithsonian throughout the rest of his career. By early 1886 he had commenced graduate studies at the State University of Iowa (now known as the University of Iowa) under Prof. Samuel Calvin, but was hired in June 1886 as Curator of the Cabinet of Natural History and Instructor in Natural Science and never completed a Ph.D. Nutting was promoted to Assistant Professor in 1888, and to Professor and Head of the Department of Systematic Zoology in 1889. His doctorate, awarded in 1926 by Cornell College, was an honorary LL.D. (Pammel, 1927; Stoner, 1927; Wickham, 1934; Taylor, 1943; Stromsten, 1950).

As an adult, Nutting (Fig. 1) was sturdy and strong but short in stature (about 5 1/2 feet tall) (Taylor, 1937). Ominously, he failed a physical examination for insurance as a young man because of a 'rapid heart' (Taylor, 1943). According to Taylor he was adventuresome, energetic, hard-working, organized, generous, tolerant, honest, disciplined, and not easily intimidated. Nutting loved his family, enjoyed nature, and was an avid reader. An American mid-westerner all his life, he nevertheless developed a particular passion for the sea and marine life. He was proud to have never been seasick in spite of experiencing some severe storms. Notwithstanding firm religious convictions, he was a staunch defender of Darwin (Anonymous, 1927; Pammel, 1927; Taylor, 1937, 1943).

On 10 August 1886 Nutting married Elizabeth ('Lizzie') Hersman, whom he had met at Blackburn College. Lizzie died a few days after the birth of their daughter, Elizabeth Hersman, on 30 April 1891. Six years later, after seeking his young daughter's approval, he married Eloise Willis on 16 June 1897. Their two sons, Willis (b. 1900) and Charles (b. 1906), became highly respected academics. Lizzie and later Eloise assisted him in his research and accompanied him on collecting expeditions (Nutting, 1889a, 1919).

Nutting and the UI Museum of Natural History

The 'Cabinet of Natural History' at the University of Iowa (UI), second oldest museum in the United States west of the Mississippi River, was founded in 1858. The initial focus of this museum was geology, but its scientific scope was broadened in the 1870s by Samuel Calvin, Curator and Professor of Natural Science. Its primary purpose at the time was to serve as a teaching resource for the natural sciences, following a model established by Louis Agassiz at the Museum of Comparative Zoology. In 1885 the Cabinet was moved from the Old Capitol building to the new Science Hall (later Calvin Hall) (Schrimper, 1982, 1992).

In 1887, a year after Nutting was appointed Curator, the 'cabinet' was re-named the Museum of Natural History (MNH). He advocated that such museums should serve students, first and foremost (Nutting, 1917), but he also recognized their value in research and in broader public education. Under his leadership the academic

Figure 1. Charles Cleveland Nutting, 1897 (courtesy Archives, Special Collections Department, University of Iowa Libraries, Iowa City, Iowa).

reputation of the MNH grew rapidly, bringing national and international recognition to the university. It also became a prime attraction locally for its exhibits and educational programs (Schrimper, 1992).

Nutting was skillful in acquiring important new collections and developing innovative exhibits. For example, he secured the W.T. Hornaday collection of birds and mammals at the outset of his career, in 1886 (Schrimper, 1982). In 1898 he obtained the skeleton of a North Atlantic Right Whale, taken off North Carolina (Thomson, 1987). Later he worked with Homer Dill of the MNH in replacing static cases of specimens with new galleries. Most notable of these was the acclaimed Laysan Island Cyclorama, opened in 1914. Surrounding the visitor on all sides, it comprised a multidisciplinary diorama portraying the bird rookeries and general ecology of Laysan. Its main theme was conservation of a unique oceanic island ecosystem. Nutting conceived of the display after visiting Laysan in 1902 during a cruise of the U.S. Fisheries Steamer 'Albatross.' The Laysan Island Cyclorama, the right whale skeleton, and a Hornaday diorama of orangutans are still on exhibit at the museum. Under Nutting's directorship, the museum also hosted public presentations by scientists and explorers. An enthusiastic speaker, he gave

lectures on his own expeditions to promote understanding of science and appreciation of research (Pammel, 1927; Taylor, 1943; Schrimper, 1992). An ability to popularize his work made Nutting effective as a fund-raiser for the museum.

By the beginning of the 20th century, with cramped quarters, rapidly growing collections, limited exhibition space, and irreplaceable specimens at risk from fire and mold, Nutting convinced the university administration to erect a new building for the museum and the natural science departments. He envisioned a grand edifice with a museum at its core, and with the natural science departments in either wing (Taylor, 1943). A fine stone building (the Natural Sciences Building, renamed Macbride Hall in 1934), meeting Nutting's plan and designed to be fireproof, was completed in 1907. To his dismay, the museum and Zoology were thereupon assigned to either end of the building, while the center accommodated a temporary library and an auditorium. Geology and Botany remained in Science Hall (Schrimper, 1982, 1992). It would be four decades before the library vacated the building, and the auditorium is still there. The Museum of Natural History now occupies much of Macbride Hall, included in the National Register of Historic Places in the United States. Although named in honor of Thomas Macbride, a distinguished botanist and president at Iowa, 'More than any other individual at the University, Nutting was responsible for the construction of the Natural Sciences Building …' (Schrimper, 1992).

Nutting is especially remembered at Iowa for undertaking a number of successful international expeditions. Most were marine-oriented trips to obtain specimens for teaching, exhibits, and research. They also provided field experience for students and faculty at a university in the American heartland. Notable trips lead or co-lead by Nutting included those to the Bay of Fundy (1890), Manitoba (1891), the Bahamas and vicinity (1893), Barbados and Antigua (1918), and Fiji and New Zealand (1922) (Stoner, 1927; Taylor, 1943; Stromsten, 1950; Schrimper, 1992). Popular narratives were provided for the latter three (Nutting, 1895a, 1919, 1924). His expeditions were meticulously planned and executed, and each member of the field party was assigned responsibility for one or more tasks (Taylor, 1937, 1943). In addition to his own trips, Nutting was invited as scientist responsible for invertebrates on the 'Albatross' Expedition of 1902 to the Hawaiian Islands (Wickham, 1934).

The most noteworthy of his expeditions was to the Bahamas, Cuba, the Florida Keys, and Dry Tortugas in 1893. Nutting and two instructor-assistants led a scientific party of 21, including students, on a trip lasting 83 d (Nutting 1895a; Schrimper, 1992). Fully 1/3 of his scientific crew was female. They departed Baltimore aboard the 'Emily E. Johnson,' a 116 ton, 95-foot, two-masted double-topsail centerboard schooner. This sailing vessel, chosen instead of a steamer in the interests of economy (Nutting, 1901f), was modified for biological work in advance of the cruise. According to Nutting (1895a), 'It is doubtful if any skipper ever started on a 3 months' cruise with a more inexperienced lot of 'land-lubbers' than Captain James B. Flowers had on board the 'Emily E. Johnson…' However, the expedition was a resounding success, with over 15 000 specimens obtained for study and exhibit. Collections provided material for decades of laboratory study, exhibits, and research, including Nutting's (1900, 1904a, 1915) monographs on American Hydroids. Remarkably, for a cruise of that duration, 'There was no accident or misfortune of any kind, and no sickness except the inevitable sea-sickness' (Nutting, 1901f).

The success of the Bahamas expedition lead Nutting to undertake another, 25 years later, to Barbados, Antigua, and nearby islands in the Lesser Antilles. A scientific party of 19, including Nutting's wife Eloise and sons Willis and Carl, departed Iowa City on 23 April 1918 and returned that summer (Nutting, 1919). His final expedition, to Fiji and New Zealand with a team of six, occurred from 14 May to 7 September 1922 (Nutting, 1924; Stromsten, 1950).

Research on hydroids and other cnidarians

An avid naturalist, Nutting published on a wide range of subjects, from protozoans to birds, from bioluminescence to the aurora borealis, and from Darwin and evolutionary theory to his own expeditions (Taylor, 1937, 1943). He developed an especial appreciation for marine biology, and for

Cnidaria (Stoner, 1927), as an outcome of a field trip to the Bahamas with wife Lizzie in 1888 (Wickham, 1934). In all, 31 of his publications dealt with cnidarians. Some 20 of these were on hydroids, and research on them largely established his scientific reputation (Taylor, 1943).

Nutting's initial publications on cnidarians comprised an anatomical study of six species of octocorals collected in the Bahamas during 1888 (Nutting, 1889a), and an abstract on nematocyst research (Nutting, 1889b). His interest in hydroids arose as a result of the Bahamas expedition of 1893 (Nutting, 1895a). In spring and summer of 1895 he visited Europe to advance his knowledge of the group. April and the first half of May were spent at The Laboratory, Marine Biological Association of the United Kingdom, in Plymouth. He then went to Italy and the Stazione Zoologica di Napoli. As with many 19th and early 20th century American marine zoologists, his interest in marine invertebrates was encouraged by the Agassiz family. In 1896, Alexander Agassiz provided Nutting with research facilities at his laboratory at Newport, Rhode Island. During summers from 1897 to 1899, Nutting worked at the U.S. Fish Commission Laboratory in Woods Hole, Massachusetts.

Nutting's contributions on hydroids began modestly. Several species collected during the Bahamas expedition, including his first new species of hydroid, were addressed briefly in the cruise narrative (Nutting, 1895a). An overview of reproduction in plumularioids (Nutting, 1895b) was also published that year. The following year he gave an account of several species from Plymouth, England (Nutting, 1896), three of them being new (Table 1). These were illustrated and more fully described 2 years later (Nutting, 1898a), but their names are available from the 1896 paper. His next works were identical reports, in two journals, on nematophores of plumularioids (Nutting, 1898b, 1898c). As the decade ended he published on hydroids from Alaska and Puget Sound (Nutting, 1899) sent to him by Trevor Kincaid of the University of Washington.

At the behest of G. Brown Goode of the Smithsonian, Nutting had begun work in the 1890s on American Hydroids, Part I (Nutting, 1900). 'American' was used in the broad sense, encompassing both North and South America. For this tome, Nutting examined large collections of specimens from the United States National Museum and the museum at Yale University, as well as his own material from the Bahamas Expedition. He began Part I, on plumularioids, with detailed reviews of the morphology, development, systematics, and bathymetric distribution of the group (Nutting, 1900). This was followed by a systematic section, with synonymy lists, descriptions, distribution records, and illustrations of 121 nominal species. Four genera and 55 species were described as new (Table 1). A model of sound monographic work, it covered what is now recognized as four families (Kirchenpaueriidae, Plumulariidae, Halopterididae, and Aglaopheniidae).

The following year, Nutting reported on hydroids of the Woods Hole region, Massachusetts (Nutting, 1901a), based on his summer studies in New England between 1896 and 1899, and on hydroids from the Harriman Alaska Expedition (Nutting, 1901c). He also published various abstracts and correspondence dealing with Cnidaria over that decade (Nutting, 1901b–e, 1904b, 1908b, 1910f).

American hydroids, Part II (Nutting, 1904a), was published 4 years after Part I. It followed the same format and met the same high standards as the first volume. Of 131 nominal species assigned today to Sertulariidae and Syntheciidae, 32 were described as new. Soon after, Nutting described the hydroids from a cruise of the 'Albatross' to Hawaii in 1902 (Nutting, 1905). That paper remains the primary systematic reference on hydroids of the region.

Nutting then interrupted his monographic work on American hydroids to carry out research on several large collections of alcyonarians. He published on material from Hawaii, obtained by the 'Albatross' in 1902 (Nutting, 1908a), and on specimens from the coast of California (Nutting, 1909). Those reports were followed by six monographs on extensive collections acquired around Indonesia by the Siboga Expedition (Nutting, 1910a–e, 1911). A final paper on alcyonarians provided descriptions of the fauna collected by the 'Albatross' around Japan and elsewhere in the northwest Pacific (Nutting, 1913). In all, he described and named some nine new genera and 206 new species of Alcyonaria. Nutting's alcyonarian work seems to have survived the test of time

Table 1. Available names of nominal species, genera, and families of hydroids authored by C.C. Nutting

Original name	Locality	Current name
Species		
Sertularella quadrata Nutting, 1895	Cuba: off Havana	*Sertularella quadrata* Nutting, 1895
Plumularia alleni Nutting, 1896	UK: Plymouth Sound	?*Plumularia alleni* Nutting, 1896
Opercularella hispida Nutting, 1896	UK: Plymouth	*Calycella hispida* Nutting, 1896
Eudendrium album Nutting, 1896	UK: Millbay Channel	*Eudendrium album* Nutting, 1896
Campanularia kincaidi Nutting, 1899	USA: Washington, Puget Sound	*Clytia kincaidi* (Nutting, 1899)
Campanularia lineata Nutting, 1899	USA: Washington, Puget Sound	*Campanularia groenlandica* Levinsen, 1893 (?)
Halecium corrugatum Nutting, 1899	USA: Washington, Puget Sound	*Halecium corrugatum* Nutting, 1899
Halecium geniculatum Nutting, 1899[a]	USA: Washington, Puget Sound	*Halecium washingtoni* Nutting, 1901
Hydrallmania distans Nutting, 1899	USA: Washington, Puget Sound	*Hydrallmania distans* Nutting, 1899
Aglaophenia allmani Nutting, 1900	USA: Florida, Florida Keys	*Macrorhynchia allmani* (Nutting, 1900)
Aglaophenia aperta Nutting, 1900	Cuba: off Havana	*Aglaophenia apocarpa* Allman, 1877
Aglaophenia bicornuta Nutting, 1900	Cuba: off Havana	*Aglaophenia bicornuta* Nutting, 1900
Aglaophenia contorta Nutting, 1900	USA: Florida, Florida Keys	*Aglaophenia tridentata* Versluys, 1899
Aglaophenia cristifrons Nutting, 1900	Cuba: off Havana	*Aglaophenia apocarpa* Allman, 1877
Aglaophenia dubia Nutting, 1900	USA: Florida, Florida Keys	*Aglaophenia dubia* Nutting, 1900
Aglaophenia elegans Nutting, 1900[b]	USA: Florida, Florida Keys	*Aglaophenia apocarpa* Allman, 1877
Aglaophenia flowersi Nutting, 1900	USA: Florida, Florida Keys	*Aglaophenia dubia* Nutting, 1900
Aglaophenia latirostris Nutting, 1900	Brazil: no locality given	*Aglaophenia latirostris* Nutting, 1900
Aglaophenia mammillata Nutting, 1900	USA: North Carolina	*Aglaophenia latecarinata* Allman, 1877
Aglaophenia minima Nutting, 1900	Bahamas: Little Cat Is.	*Aglaophenia latecarinata* Allman, 1877
Aglaophenia octocarpa Nutting, 1900	Mexico: Cabo San Lucas	*Aglaophenia octocarpa* Nutting, 1900
Aglaophenia rathbuni Nutting, 1900	Brazil: Caravellas	*Aglaophenia rhynchocarpa* Allman, 1877
Aglaophenopsis distans Nutting, 1900	USA: Georgia	*Aglaophenopsis distans* Nutting, 1900
Aglaophenopsis verrilli Nutting, 1900	USA: off New York	*Aglaophenopsis verrilli* Nutting, 1900
Antennopsis distans Nutting, 1900	Cuba: off Havana	*Nemertesia distans* (Nutting, 1900)
Antennopsis longicorna Nutting, 1900	Cuba: off Havana	*Nemertesia longicorna* (Nutting, 1900)
Antennopsis nigra Nutting, 1900	Cuba: off Havana	*Nemertesia nigra* (Nutting, 1900)
Antennularia americana Nutting, 1900	USA: Rhode Island	*Nemertesia americana (Nutting, 1900)*
Antennularia geniculata Nutting, 1900	USA: Georgia	*Nemertesia geniculata* (Nutting, 1900)
Antennularia pinnata Nutting, 1900	USA: Massachusetts	*Nemertesia pinnata* (Nutting, 1900)
Antennularia rugosa Nutting, 1900	USA: Massachusetts	*Nemertesia rugosa* (Nutting, 1900)
Calvinia mirabilis Nutting, 1900	USA: Georgia/Florida	*Calvinia mirabilis* Nutting, 1900
Cladocarpus carinatus Nutting, 1900	USA: Florida	*Cladocarpus carinatus* Nutting, 1900
Cladocarpus flexuosus Nutting, 1900	USA: Alabama	*Cladocarpus flexuosus* Nutting, 1900
Cladocarpus grandis Nutting, 1900	USA: Florida	*Cladocarpus grandis* Nutting, 1900
Cladocarpus obliquus Nutting, 1900	Cuba: off Havana	*Cladocarpus obliquus* Nutting, 1900
Cladocarpus septatus Nutting, 1900	USA: off Maryland	*Cladocarpus septatus* Nutting, 1900
Diplopteron grande Nutting, 1900	USA: Georgia/Florida	*Schizotricha grande* (Nutting, 1900) (?)
Diplopteron longipinna Nutting, 1900	USA: Florida, Florida Keys	*Schizotricha longipinna* (Nutting, 1900) (?)
Diplopteron quadricorne Nutting, 1900	Cuba: off Havana	*Schizotricha quadricorne* (Nutting, 1900) (?)
Halicornaria longicauda Nutting, 1900	Panama: off Colon	*Gymnangium longicaudum* (Nutting, 1900)
Halicornaria variabilis Nutting, 1900	Bahamas: Little Cat Is.	*Gymnangium speciosum* (Allman, 1877)
Hippurella longicarpa Nutting, 1900	St. Vincent (West Indies)	*Hippurella longicarpa* Nutting, 1900
Lytocarpus clarkei Nutting, 1900	Bahamas: Little Cat Is.	*Macrorhynchia clarkei* (Nutting, 1900)
Lytocarpus curtus Nutting, 1900	Bahamas: Little Cat Is.	*Macrorhynchia curta* (Nutting, 1900)
Lytocarpus furcatus Nutting, 1900	Bahamas: Little Cat Is.	*Macrorhynchia furcata* (Nutting, 1900)

Continued on p. 19

Table 1. (Continued)

Original name	Locality	Current name
Monotheca margaretta Nutting, 1900	Bahamas: Little Cat Is.	*Monotheca margaretta* Nutting, 1900
Plumularia alternata Nutting, 1900	Bahamas: Barracuda Rocks	*Halopteris alternata* (Nutting, 1900)
Plumularia altitheca Nutting, 1900	USA: Georgia/Florida	*Plumularia altitheca* Nutting, 1900 (?)
Plumularia clarkei Nutting, 1900	Cuba: off Havana	*Halopteris clarkei* (Nutting, 1900)
Plumularia corrugata Nutting, 1900	South Atlantic Ocean, E of Brazil	*Plumularia setacea* (Linnaeus, 1758)
Plumularia dendritica Nutting, 1900	Bahamas: Little Cat Is.	*Dentitheca dendritica* (Nutting, 1900)
Plumularia floridana Nutting, 1900	USA: Florida	*Plumularia floridana* Nutting, 1900
Plumularia goodei Nutting, 1900	USA: California, Santa Barbara	*Plumularia goodei* Nutting, 1900
Plumularia inermis Nutting, 1900	Bahamas: Barracuda Rocks	*Ventromma halecioides* (Alder, 1859)
Plumularia palmeri Nutting, 1900	USA: California, San Diego	*Plumularia setacea* (Linnaeus, 1758) (?)
Plumularia paucinoda Nutting, 1900	Cuba: off Havana	*Plumularia paucinoda* Nutting, 1900
Plumularia profunda Nutting, 1900	USA: Georgia	*Schizotricha profunda* (Nutting, 1900)
Plumularia virginiae Nutting, 1900	USA: California, Santa Barbara	*Plumularia virginiae* Nutting, 1900
Polyplumularia armata Nutting, 1900	USA: Georgia	*Polyplumaria armata* Nutting, 1900 (?)
Schizotricha dichtoma Nutting, 1900	USA: Florida, Pourtales Plateau	*Schizotricha dichtoma* Nutting, 1900
Schizotricha parvula Nutting, 1900	USA: Georgia	*Schizotricha parvula* Nutting, 1900
Thecocarpus benedicti Nutting, 1900	USA: Georgia	*Lytocarpia benedicti* (Nutting, 1900)
Thecocarpus normani Nutting, 1900	USA: Georgia	*Lytocarpia normani* (Nutting, 1900)
Campanularia reduplicata Nutting, 1901	USA: Alaska, Yakutat	*Campanularia volubilis* (Linnaeus, 1758)
Campanularia regia Nutting, 1901	USA: Alaska, Orca	*Bonneviella regia* (Nutting, 1901)
Campanularia ritteri Nutting, 1901	USA: Alaska, Juneau	*Campanularia ritteri* Nutting, 1901
Campanulina rugosa Nutting, 1901	USA: Alaska, Juneau	*Opercularella rugosa* (Nutting, 1901), comb. nov.
Coryne brachiata Nutting, 1901	USA: Alaska, Yakutat Bay	*Coryne brachiata* Nutting, 1901
Garveia annulata Nutting, 1901	USA: Alaska, Yakutat/Sitka	*Garveia annulata* Nutting, 1901
Gonothyraea inornata Nutting, 1901	USA: Alaska, Yakutat Bay	*Laomedea inornata* (Nutting, 1901)
Grammaria immersa Nutting, 1901	USA: Alaska, Kodiak	*Grammaria immersa* Nutting, 1901
Halecium ornatum Nutting, 1901	USA: Alaska, Glacier Bay	*Halecium ornatum* Nutting, 1901
Halecium reversum Nutting, 1901	USA: Alaska, Juneau	*Halecium reversum* Nutting, 1901
Halecium robustum Nutting, 1901[c]	USA: Alaska, Glacier Bay	*Halecium harrimani* Nutting, 1901
Halecium speciosum Nutting, 1901	USA: Alaska, Yakutat Bay	*Halecium speciosum* Nutting, 1901
Lafoea adhaerens Nutting, 1901	USA: Alaska, Kodiak Harbor	*Lafoea adhaerens* Nutting, 1901
Obelia borealis Nutting, 1901	USA: Alaska, Yakutat	*Obelia longissima* auct.
Obelia dubia Nutting, 1901	USA: Alaska, Orca	*Obelia dubia* Nutting, 1901
Sertularella saccata Nutting, 1901	USA: Alaska, Popof Is.	*Sertularella rugosa* (Linnaeus, 1758)
Thuiaria coei Nutting, 1901	USA: Alaska, Dutch Harbor	*Abietinaria coei* (Nutting, 1901) (?)
Thuiaria costata Nutting, 1901	USA: Alaska, Yakutat	*Abietinaria costata* (Nutting, 1901)
Thuiaria dalli Nutting, 1904	USA: Alaska, Yakutat	*Sertularia dalli* (Nutting, 1904)
Thuiaria elegans Nutting, 1901[d]	USA: Alaska, Glacier Bay	*Abietinaria kincaidi* (Nutting, 1901)
Tubularia harrimani Nutting, 1901	USA: Alaska, Orca	*Tubularia harrimani* Nutting, 1901
Halecium harrimani Nutting, 1901	USA: Alaska, Glacier Bay	*Halecium harrimani* Nutting, 1901 (?)
Halecium washingtoni Nutting, 1901	USA: Washington, Puget Sound	*Halecium washingtoni* Nutting, 1901
Thuiaria kincaidi Nutting, 1901	USA: Alaska: Glacier Bay	*Abietinaria kincaidi* (Nutting, 1901)
Campanularia edwardsi Nutting, 1901	USA: Massachusetts	*Clytia edwardsi* (Nutting, 1901)
Campanularia minuta Nutting, 1901	USA: Massachusetts	*Clytia minuta* (Nutting, 1901)
Clytia grayi Nutting, 1901	USA: Massachusetts	*Clytia hemisphaerica* (Linnaeus, 1767)
Lovenella grandis Nutting, 1901	USA: Rhode Island	*Lovenella grandis* Nutting, 1901
Abietinaria alexanderi Nutting, 1904	USA: Alaska, Aleutian Islands	*Abietinaria alexanderi* Nutting, 1904

Continued on p. 20

Table 1. (Continued)

Original name	Locality	Current name
Abietinaria amphora Nutting, 1904	USA: Alaska/Washington	*Abietinaria amphora* Nutting, 1904
Abietinaria gracilis Nutting, 1904	USA: Alaska, Aleutian Is.	*Abietinaria gracilis* Nutting, 1904
Dictyocladium flabellum Nutting, 1904	USA: Alaska, Aleutian Is.	?*Dictyocladium flabellum* Nutting, 1904
Diphasia paarmanni Nutting, 1904	USA: Florida	*Diphasia paarmanni* Nutting, 1904
Diphasia pulchra Nutting, 1904	USA: Washington, St. of Georgia	*Abietinaria pulchra* (Nutting, 1904)
Diphasia tropica Nutting, 1904	Bahamas: Little Cat Is.	*Diphasia tropica* Nutting, 1904
Selaginopsis hartlaubi Nutting, 1904	USA: Alaska, Pribilof Islands	*Selaginopsis hartlaubi* Nutting, 1904
Selaginopsis ornata Nutting, 1904	USA: Alaska, Aleutian Islands	*Selaginopsis cylindrica* (Clark, 1877)
Selaginopsis plumiformis Nutting, 1904	USA: Alaska, off Nunivak Is.	*Selaginopsis plumiformis* Nutting, 1904
Sertularella areyi Nutting, 1904	Cuba: off Havana	*Sertularella areyi* Nutting, 1904
Sertularella complexa Nutting, 1904	USA: Alaska, Pribilof Is.	*Sertularella complexa* Nutting, 1904
Sertularella elegans Nutting, 1904	USA: Alaska, Aleutian Islands	*Symplectoscyphus elegans* (Nutting, 1904)
Sertularella hartlaubi Nutting, 1904	Jamaica	*Sertularella hartlaubi* Nutting, 1904
Sertularella levinseni Nutting, 1904	USA: Alaska, Aleutian Islands	*Symplectoscyphus levinseni* (Nutting, 1904)
Sertularella magna Nutting, 1904	USA: Alaska, Aleutian Islands	*Sertularella magna* Nutting, 1904
Sertularella megastoma Nutting, 1904	Mexico: Yucatan	*Sertularella megastoma* Nutting, 1904
Sertularella meridionalis Nutting, 1904	Chile: near Hanover Is.	*Symplectoscyphus milneanus* (d'Orbigny, 1846)
Sertularella minuta Nutting, 1904	USA: Alaska, Aleutian Islands	*Symplectoscyphus minutus* (Nutting, 1904)
Sertularella solitaria Nutting, 1904	Bahamas: Eleuthera/Little Cat Is.	*Sertularella solitaria* Nutting, 1904
Sertularella tanneri Nutting, 1904	USA: Washington, Juan de Fuca St.	*Sertularella tanneri* Nutting, 1904
Sertularia challengeri Nutting, 1904	Brazil: Bahia	*Tridentata marginata* (Kirchenpauer, 1864)
Sertularia flowersi Nutting, 1904	Cuba: off Havana	*Tridentata flowersi* (Nutting, 1904)
Sertularia mayeri Nutting, 1904	Bahamas: Eleuthera/Little Cat Is.	*Dynamena disticha* (Bosc, 1802)
Sertularia pourtalesi Nutting, 1904	USA: Florida Keys	*Dynamena disticha* (Bosc, 1802)
Sertularia rathbuni Nutting, 1904	Gulf of Mexico	*Dynamena dalmasi* (Versluys, 1899)
Sertularia stookeyi Nutting, 1904	Bahamas: Bahama Banks	*Tridentata distans* (Lamouroux, 1816)
Sertularia versluysi Nutting, 1904	Bermuda	*Tridentata marginata* (Kirchenpauer, 1864)
Synthecium rectum Nutting, 1904	Cuba: off Havana	*Synthecium tubithecum* (Allman, 18 77)
Synthecium robustum Nutting, 1904	Chile: Strait of Magellan	*Synthecium robustum* Nutting, 1904
Thuiaria immersa Nutting, 1904	Greenland	*Thuiaria laxa* Allman, 1874
Thuiaria kurilae Nutting, 1904	USA: Alaska, Aleutian Is.	*Amphisbetia kurilae* (Nutting, 1904)
Antennella complexa Nutting, 1905	USA: Hawaii, Pailolo Channel	*Corhiza complexa* (Nutting, 1905)
Balea mirabilis Nutting, 1905	USA: Hawaii, Pailolo Channel	*Balella mirabilis* (Nutting, 1905)
Campanularia eloisa Nutting, 1905	USA: Hawaii, Maui/Molokai Is.	*Rhizocaulus eloisa* (Nutting, 1905)
Corydendrium corrugatum Nutting, 1905	USA: Hawaii, Oahu/Maui/Kaui Is.	*Corydendrium corrugatum* Nutting, 1905
Corydendrium minor Nutting, 1905	USA: Hawaii, Maui Is.	*Turritopsis minor* (Nutting, 1905)
Cryptolaria operculata Nutting, 1905	USA: Hawaii, Pailolo Channel	*Stegolaria operculata* (Nutting, 1905)
Cryptolaria symmetrica Nutting, 1905	USA: Hawaii, Maui/Molokai Is.	*Acryptolaria symmetrica* (Nutting, 1905)
Diphasia palmata Nutting, 1905	USA: Hawaii, Molokai Is.	*Diphasia palmata* Nutting, 1905
Halecium scandens Nutting, 1905	USA: Hawaii, Laysan Is.	*Halecium scandens* Nutting, 1905
Halicornaria briani Nutting, 1905	USA: Hawaii, Kauai Is.	*Gymnangium briani* (Nutting, 1905)
Halicornaria flava Nutting, 1905	USA: Hawaii, Laysan Is.	*Gymnangium hians* (Busk, 1852)
Hydrodendrium gorgonoides Nutting, 1905	USA: Hawaii, Kauai Channel	*Hydractinia gorgonoides* (Nutting, 1905)
Lafoea contorta Nutting, 1905	USA: Hawaii, Laysan & Pailolo Ch.	*Cryptolarella contorta* (Nutting, 1905)
Lictorella cervicornis Nutting, 1905	USA: Hawaii, Pailolo Channel	*Zygophylax cervicornis* (Nutting, 1905)
Lytocarpus balei Nutting, 1905	USA: Hawaii, Molokai Is.	*Macrorhynchia balei* (Nutting, 1905)
Lytocarpus hawaiensis Nutting, 1905	USA: Hawaii, Molokai/Maui Is.	*Macrorhynchia hawaiensis* (Nutting, 1905)

Continued on p. 21

Table 1. (Continued)

Original name	Locality	Current name
Lytocarpus similis Nutting, 1905	USA: Hawaii, Molokai Is.	*Macrorhynchia similis* (Nutting, 1905)
Monostaechas fisheri Nutting, 1905	USA: Hawaii, Maui/Laysan Is.	*Monostaechas fisheri* Nutting, 1905
Opercularella longicauda Nutting, 1905	USA: Hawaii, Pailolo Channel	*Opercularella longicauda* Nutting, 1905
Plumularia delicata Nutting, 1905	USA: Hawaii, Maui Is.	*Plumularia delicata* Nutting, 1905
Plumularia jordani Nutting, 1905	USA: Hawaii, Laysan Is.	*Plumularia jordani* Nutting, 1905
Plumularia milleri Nutting, 1905	USA: Hawaii, Maui Is.	*Plumularia milleri* Nutting, 1905
Stegopoma gilberti Nutting, 1905	USA: Hawaii, Pailolo Channel	*Modeeria rotunda* (Quoy & Gaimard, 1827)
Stegopoma gracilis Nutting, 1905	USA: Hawaii, Maui/ Kaui Is.	*Modeeria rotunda* (Quoy & Gaimard, 1827)
Stegopoma plumicola Nutting, 1905	USA: Hawaii, Laysan Is.	*Stegopoma plumicola* Nutting, 1905
Sertularella crenulata Nutting, 1905	USA: Hawaii, Molokai Is.	*Sertularella crenulata* Nutting, 1905
Sertularella torreyi Nutting, 1905	USA: Hawaii, Molokai Is.	*Sertularella diaphana* (Allman, 1885)
Sertularia snyderi Nutting, 1905	USA: Hawaii, Laysan Is.	*Tridentata snyderi* (Nutting, 1905)
Thecocarpus niger Nutting, 1905	USA: Hawaii, Laysan Is.	*Lytocarpia niger* (Nutting, 1905)
Bonneviella ingens Nutting, 1915	Russia: Kuril Islands	*Bonneviella ingens* Nutting, 1915
Bonneviella superba Nutting, 1915	Bering Sea	*Bonneviella superba* Nutting, 1915
Campanularia brevicaulis Nutting, 1915	Cuba: off Havana	*Campanularia brevicaulis* Nutting, 1915
Campanularia spiralis Nutting, 1915	Russia: Kamchatka	*Orthopyxis integra* (Macgillivray, 1842)
Clytia sargassicola Nutting, 1915	Bahamas: off Eleuthera Is.	*Orthopyxis sargassicola* (Nutting, 1915)
Acryptolaria normani Nutting, 1927[e]	Philippines: Lauis Point	*Acryptolaria tizardensis* Kirkpatrick, 1890
Aglaophenia triramosa Nutting, 1927	South China Sea, off Hong Kong	*Aglaophenia triramosa* Nutting, 1927
Antennella biarmata Nutting, 1927	Philippines: Davao Bay	*Antennella biarmata* Nutting, 1927
Antennella recta Nutting, 1927	South China Sea, off Hong Kong	*Antennella recta* Nutting, 1927
Antennopsis pacifica Nutting, 1927	Philippines: Sirun Is.	*Nemertesia pacifica* (Nutting, 1927)
Dictyocladium aberrans Nutting, 1927	Indonesia: Sibuko Bay	*Dictyocladium aberrans* Nutting, 1927
Diphasia inornata Nutting, 1927	Philippines: Hermanos Is.	*Diphasia inornata* Nutting, 1927
Halicornaria magnirostris Nutting, 1927	Philippines: Jolo	*Gymnangium magnirostre* (Nutting, 1927)
Halicornaria tenuirostris Nutting, 1927	Philippines: Linao Point	*Gymnangium tenuirostre* (Nutting, 1927)
Hebella spiralis Nutting, 1927	Philippines: Surigao Strait	*Hebellopsis scandens* (Bale, 1888)
Obelia thornelyi Nutting, 1927	Philippines: Davao	*Clytia thornelyi* (Nutting, 1927)
Plumularia camarata Nutting, 1927	Philippines: Observation Is.	*Plumularia camarata* Nutting, 1927
Plumularia flabellata Nutting, 1927	Philippines: Davao Bay	*Plumularia flabellata* Nutting, 1927
Plumularia hargitti Nutting, 1927	Philippines: Jolo	*Plumularia hargitti* Nutting, 1927
Stechowia armata Nutting, 1927	Philippines: Davao Bay	*Sibogella armata* Nutting, 1927
Stegopoma dimorpha Nutting, 1927	Philippines: Balayan Bay	*Stegopoma dimorpha* Nutting, 1927
Thecocarpus balei Nutting, 1927	Philippines: Balukbaluk Is.	*Lytocarpia balei* Nutting, 1927
Genera		
Calvinia Nutting, 1900		*Calvinia* Nutting, 1900
Monotheca Nutting, 1900		*Monotheca* Nutting, 1900
Nuditheca Nutting, 1900		*Nuditheca* Nutting, 1900
Thecocarpus Nutting, 1900		*Lytocarpia* Kirchenpauer, 1872
Balea Nutting,1905[f]		*Balella* Stechow, 1919
Hydrodendrium Nutting, 1905		*Hydractinia* van Beneden, 1841
Stechowia Nutting, 1927[g]		*Sibogella* Billard, 1911
Families		
Hydrodendridae Nutting, 1905		Hydractiniidae L. Agassiz, 1862
Tubidendridae Nutting, 1905[h]		Balellidae Stechow, 1919

Continued on p. 22

Table 1. (Continued)

Types of Nutting's nominal species, except for certain replacement names, are in collections at the National Museum of Natural History, Smithsonian Institution. Names used in unpublished manuscripts have not been included.

[a]*Halecium geniculatum* Nutting, 1899 is an invalid junior primary homonym of *Halecium geniculatum* Norman, 1867 [= *Halecium halecinum* (Linnaeus, 1758)] and was renamed *H. washingtoni* by Nutting (1901b).

[b]*Aglaophenia elegans* Nutting, 1900 is an invalid junior primary homonym of *Aglaophenia elegans* Lamouroux, 1816. Fraser (1944) replaced Nutting's name of the species with *A. raridentata*. It is now considered conspecific with *A. apocarpa* Allman, 1877.

[c]*Halecium robustum* Nutting, 1901 is an invalid junior primary homonym of *Halecium robustum* Verrill, 1873 and *Halecium robustum* Pieper, 1884, and was renamed *Halecium harrimani* by Nutting (1901b).

[d]*Thuiaria elegans* Nutting, 1901 is an invalid junior primary homonym of *Thuiaria elegans* Kirchenpauer, 1884, and was renamed *Thuiaria kincaidi* by Nutting (1901b).

[e]Synonymy of *Acryptolaria normani* Nutting, 1927 with *Acryptolaria tizardensis* Kirkpatrick, 1890 according to Peña Cantaro et al. (in press).

[f]*Balea* Nutting, 1905, an invalid junior homonym of *Balea* Gray, 1824 (Mollusca), has been replaced by the name *Balella* Stechow, 1919.

[g]*Stechowia* Nutting, 1927 is an invalid junior homonym of *Stechowia* Poche, 1914, and a junior synonym of *Sibogella* Billard, 1911.

[h]Tubidendridae Nutting, 1905 predates Balellidae Stechow, 1919, but is invalid because it was not based on a name then valid for a contained genus (Art. 11.7, ICZN).

less-well than that on hydroids. Hjalmar Broch of Norway, who also worked on both groups, was critical of Nutting's studies on alcyonarians but decidedly positive about his monographs on hydroids (Taylor, 1937).

Upon completion of his research on Alcyonaria, Nutting returned to American Hydroids. Section III, on Campanulariidae and Bonneviellidae (Nutting, 1915), covered 86 nominal species, with five described as new. Priorities other than hydroids were now claiming much of Nutting's time at the university, and it proved to be the final volume of the series. He was also unable to work through materials collected during the Barbados-Antigua Expedition of 1918 (Fraser, 1944) and the Fiji-New Zealand Expedition of 1922.

His last paper (Nutting, 1927), published posthumously, was on hydroids collected by the 'Albatross' in the Philippine region during 1907–1910. Some 54 species were reported, eight of them new. It complemented an earlier work on Philippine hydroids by Charles Wesley Hargitt of Syracuse University (Hargitt, 1924). With the careers of Nutting and Hargitt at an end by 1927, Charles McLean Fraser remained as the only active hydroid taxonomist working in the Americas at the time.

Several manuscripts on hydroids by Nutting were never published. One was his initial work on the group, based on specimens from the Bahamas Expedition of 1893. Another was on species collected during the Northwest Pacific Expedition of the 'Albatross' in 1906. Specimens intended as types from that report are in collections at the National Museum of Natural History, Smithsonian Institution. A fourth volume of Nutting's American Hydroids, covering Lafoeidae and Hebellidae, was nearly ready for publication at the time he died in 1927 (Taylor, 1943). The plates were finished, and the text merely needed some updating. The manuscript mysteriously disappeared during a move of the Department of Zoology into a medical laboratory building in 1927, and many borrowed type specimens were lost. A critical shift of academic priorities was underway in the department and 'Taxonomy was pushed aside …' (Taylor, 1943).

In all, Nutting established two new families, seven new genera, and 175 new species of hydroids (Table 1). By far the majority of these were leptothecates, described in American Hydroids (Nutting, 1900, 1904a, 1915). According to Schuchert (1998), Nutting ranks third, after C.M. Fraser and G.J. Allman, in numbers of named hydroid species. In particular, Nutting advanced knowledge of the rich and poorly known faunas of Alaska, and of the southeastern United States and the West Indian region.

Nutting favored a conservative approach to classification and nomenclature in his hydroid research. He preferred to follow, as closely as possible, the taxonomy of two noted 19th century British workers, the Rev. Thomas Hincks and G. J. Allman of Britain (Nutting, 1901c). While acknowledging his contemporary Eberhard Stechow as a leading specialist and 'a very thorough and conscientious student of the Hydroida'

(Nutting, 1927), he disagreed with many of the revolutionary changes introduced by the prolific German worker. Nutting expressed particular concern about the confusion such changes engendered for morphologists and other biologists. The resulting instability brought systematists into disrepute, in his opinion. Although a substantial percentage of Stechow's proposals have been abandoned or modified, current hydrozoan classifications and nomenclature are closer to those proposed by him than to older systems and names followed by Nutting. Scientific knowledge of Hydrozoa was advancing rapidly at the time, and systematics had to be updated to accommodate new information. Nutting's adherence to a taxonomy that was becoming obsolete is arguably the prime criticism one might make of his otherwise splendid work. Essentially the same system was perpetuated by a graduate student of his at Iowa, Charles McLean Fraser. The influence of Nutting and Fraser is still apparent in the literature on hydroids of the Americas.

Latter years

The latter years were trying ones for the energetic professor who had contributed so much to his university, to his museum, to his discipline, and to science. Physically, he had endured chronic health problems, including rapid heartbeat, a goiter, glaucoma, and ulcers, but he did not complain and few acquaintances knew of them (Anonymous, 1927; Pammel, 1927; Taylor, 1937, 1943). Professionally, the emphasis in biology had shifted away from natural history to more experimental disciplines (Schrimper, 1992). The heyday of his museum, and even its role as a research facility, had also come and gone. A person of exceptional character, he persevered in carrying out his academic duties in spite of failing health and academic disappointments.

With Nutting's resignation as head of zoology in 1926, radical changes were taking place prior to the arrival of his replacement (geneticist Wilbur Willis Swingle). Marine collections were moved to a corridor on the ground floor of the Natural Sciences Building, and vacated space was converted into laboratories and offices (Taylor, 1937). Nutting's 5500 square-foot Invertebrate Room, with its mahogany cases of specimens, was dismantled to provide space for Home Economics (Schrimper, 1992). To a department now focused on experimental zoology, sex differentiation, and endocrinology, the museum and its collections were seen as irrelevant and an unnecessary expense. The Natural History Museum, with a new mandate on exhibits, was made a separate entity in 1927 under the directorship of Homer Dill (Stromsten, 1950).

Nutting continued as professor of zoology and museum curator, and joked that he believed he had one more expedition left in him. However, on 23 January 1927 he died of heart disease at age 68 (Stoner, 1927; Taylor, 1943). His entire academic career had been devoted to and spent at Iowa. He had served the university and its museum with energy, dedication, vision, skill, and distinction for 41 years, and his influence is still apparent on campus (especially Macbride Hall and some of its museum exhibits).

Most of Nutting's invertebrate collections, representing phyla from Porifera to Chordata, were transferred to the National Museum of Natural History in several shipments, the last of them in 1990. These collections, together with his hydroid research and the Museum of Natural History at Iowa, form primary elements of Nutting's legacy to science.

A tribute and some Patronymics

The following tribute to C.C. Nutting was written by Dayton Stoner, a university colleague and friend: 'This man truly *lived*. He gave much and he derived great satisfaction from the giving. His pioneer work in museum building and zoological investigation at the University of Iowa has been of inestimable value to the institution, its alumni and friends. As a teacher and research worker his energy and enthusiasm aroused those qualities in others as the legion of successful men and women who have come under his tutelage will testify' (Stoner, 1927).

Some scientific names honoring Nutting include the generic name *Nuttingia* Stechow, 1909 (Hydrozoa), as well as the following species names: *Myiarchus nuttingi* Ridgway, 1883 (Aves); *Oryzoborus nuttingi* Ridgway, 1884 (Aves);

Antennarius nuttingi Garman, 1896 (Pisces); *Murex nuttingi* Dall, 1896 (Gastropoda); *Xanthias nuttingi* Rathbun, 1898 (Decapoda); *Asteroschema nuttingi* Verrill, 1899 (Asteroidea); *Rhabdodermella nuttingi* Urban, 1902 (Porifera); *Gymnothorax nuttingi* Snyder, 1904 (Pisces); *Polyipnus nuttingi* Gilbert, 1905 (Pisces); *Patagiaster nuttingi* Fisher, 1906 (Asteroidea); *Pilumnus nuttingi* Rathbun, 1906 (Crustacea); *Calycella nuttingi* Hargitt, 1909 (Hydrozoa); *Turbonilla nuttingi* Dall & Bartsch, 1909 (Gastropoda); *Plumularia nuttingi* Billard, 1911 (Hydrozoa); *Sertularia nuttingi* Levinsen, 1913 (Hydrozoa); *Diphasia nuttingi* Stechow, 1913 (Hydrozoa); *Telesto nuttingi* Kükenthal, 1913 (Anthozoa); *Sertularella nuttingi* Billard, 1914 (Hydrozoa); *Astropecten nuttingi* Verrill, 1915 (Asteroidea); *Euplexaura nuttingi* Kükenthal, 1919 (Anthozoa); *Vasum globulus nuttingi* Henderson, 1919 (Gastropoda); *Exosphaeroma nuttingi* Boone, 1921 (Isopoda); *Crangon nuttingi* Schmitt, 1924 (Crustacea); *Histocidaris nuttingi* Mortensen, 1926 (Echinoidea); *Lytocarpus nuttingi* Hargitt, 1927 (Hydrozoa); *Compsometra nuttingi* Clark, 1936 (Crinoidea); *Suberogorgia nuttingi* Stiasny, 1937 (Anthozoa); *Barbatia nuttingi* (Dall, Bartsch, & Rehder, 1938) (Bivalvia); *Acabaria nuttingi* Stiasny, 1939 (Anthozoa); *Anthothela nuttingi* Bayer, 1956 (Anthozoa); *Peltastisis nuttingi* Grant, 1976 (Anthozoa); *Antipathipolyeunoa nuttingi* Pettibone, 1991 (Polychaeta); *Narella nuttingi* Bayer, 1997 (Anthozoa).

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The first description of nerves in a cnidarian: Louis Agassiz's account of 1850

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Key words: nerve net, ganglion, Romanes, Oberhäuser, Harvard college, *Bougainvillea*, *Sarsia*

Abstract

New preparations of the medusa *Bougainvillea principis* show features corresponding to those in *B. superciliaris* as described by Louis Agassiz in 1850 and are presented in context with his account. Allowing for some simplification, and one possible error, Agassiz's drawings give a good picture of the layout of the hydromedusan nervous system. On present evidence, Agassiz was the first person to discover a nervous system in any member of the Cnidaria.

Introduction

Louis Agassiz wasted no time after arriving in the United States in 1846 and within a year had assembled a group of associates from former research projects in Europe. Living together in a house in East Boston, they investigated sea shore life on the coast of Massachusetts (Agassiz, 1890; Lurie, 1960). This work resulted in an important paper dealing with hydromedusae presented verbally before the American Academy of Arts and Sciences in May 1849, and published the following year (Agassiz, 1850). The illustrations, prepared by Agassiz's lithographer Auguste Sonrel, were obviously based on drawings of living specimens and were beautifully and truthfully rendered, with none of the fanciful artistic embellishments that mar some works of natural history by writers of the period.

Included in the 1850 paper are descriptions of nerves in several medusae, including *Sarsia mirabilis* Agassiz and *Hippocrene superciliaris* Agassiz. Previously no one had claimed that coelenterates had nerves of any sort, and Agassiz's report was evidently treated with considerable skepticism. Contemporary workers found it hard to conceive of a nervous system that had no center, but consisted simply of strands or cords of nervous tissue as Agassiz described it. Romanes (1867) was among the doubters. 'I may be permitted to remark,' he writes, '… that the conclusion so positively enunciated concerning the function of the structures described has always appeared to me, as it has appeared to biologists in general, a conclusion that is certainly unwarranted by the facts'. It was not until 15 year after the publication of Agassiz's original account that nerves were again described in a hydrozoan medusa, this time by Haeckel (1865), who specifically dissociated his findings from those of Agassiz. By this time, however, Agassiz himself had had second thoughts about the nature of the structures he had described as nerves. Thus, in *Sarsia*, he explained how the folds and angles of the bell seen from certain angles gave the appearance of nerves. This he now felt to have been an erroneous impression (Agassiz, 1862). Agassiz did not include any other species in this retraction, but neither did he continue to affirm that nerves were present in them. We may assume that the retraction was meant to apply generally.

Louis Agassiz's extraordinary contributions to the development of natural history in the United States are well known. A year after he arrived in Boston, a position was created for him as

Professor of Geology at Harvard College. He had a flair for big projects, including the foundation of the Museum of Comparative Zoology at Harvard in 1860. An inspirational teacher, he established a seaside laboratory on Penikese Island where students could (as he put it) "study nature not books". Founded in 1873 (the year before he died), the lab "sparked a series of marine summer stations for teaching and research, culminating in the Marine Biological Laboratory at Woods Hole" (Dexter, 1973).

Agassiz became increasingly involved in public lecturing, teaching, writing books, and fund raising, activities that took him away from the lab bench, though he continued to oversee the work of others. It seems symbolic that in a photo of 1862 (Fig. 1A), his Oberhäuser microscope is shown tucked away and half hidden by a pile of books and papers. Affable, charming, energetic, brilliant, voluble, and eloquent in several languages, Agassiz had an apparently inexhaustible knowledge of all aspects of natural history. He was lionized by Boston society. His influence can be traced through lineages of people and institutions right down to the present day (Gladfelter, 2002).

With regard to Agassiz's microscope, pictures taken with an Oberhäuser microscope of ca.1840 (Fig. 1B) show that these instruments were capable of resolving the plexiform and nuclear layers in the retina and considerable detail in frog testis (Figs 1C and D). Given good use of the arrangements for epi- and trans-illumination, there is no reason to doubt that this instrument could be used to show nerve bundles in thin, transparent pieces of jellyfish tissue. In a previous note (Mackie, 1989) I have described the preparation methods used and suggested that, despite the relatively poor resolving power of the microscope used in this work, and despite his subsequent retraction of his claims, Agassiz's description of the distribution of nerves in *Hippocrene* was probably substantially correct, although some aspects of the description may be questionable. At the time, I had not examined this medusa, but I recently obtained some specimens of a closely related species at Friday Harbor, and looked at the layout of its nervous system with a view to checking the accuracy of Agassiz's description.

Location of nerves according to Agassiz (1850).

Agassiz saw nerves in *Sarsia* first, but on subsequent examination of several other hydromedusae he found an essentially similar pattern. The best illustrations are those for *Hippocrene* (*Bougainvillea*) (reproduced here as Figs 2A and 3A, B), which Agassiz found easiest to study "because of the facility of keeping it alive for a long time under

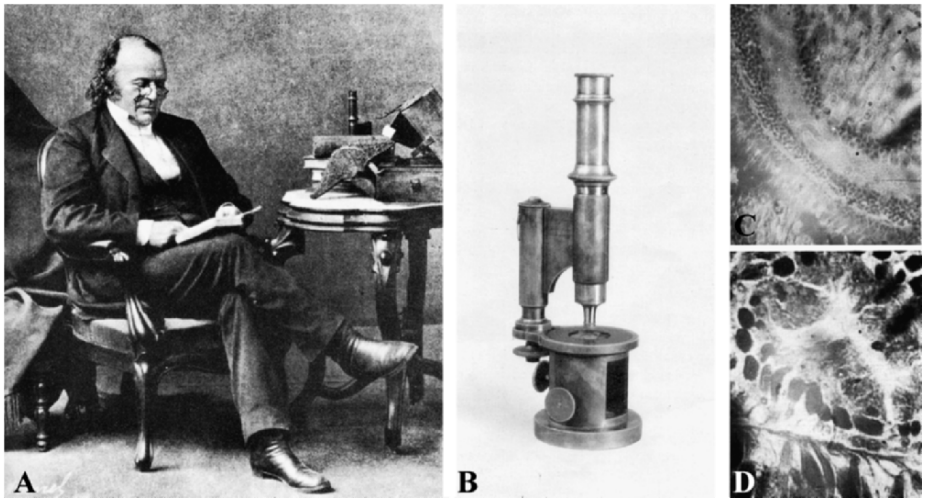

Figure 1. (A) Louis Agassiz in 1863 (from Lurie, 1960). (B) An Oberhäuser microscope of ca. 1840. For details of the nervous system, Agassiz used Oberhäuser's objective No. 8 (focal length 2.5 mm, N. (A) 0.45, magnification 42.5×) in conjunction with a No. 3 eyepiece (probably 12×), giving an overall magnification of 510×. (C) and (D) Photomicrographs taken with the microscope illustrated in (B), showing cross-sections of the retina of a fish (guppy) (C) and testis of grass frog (D). (B–D from Otto, 1970).

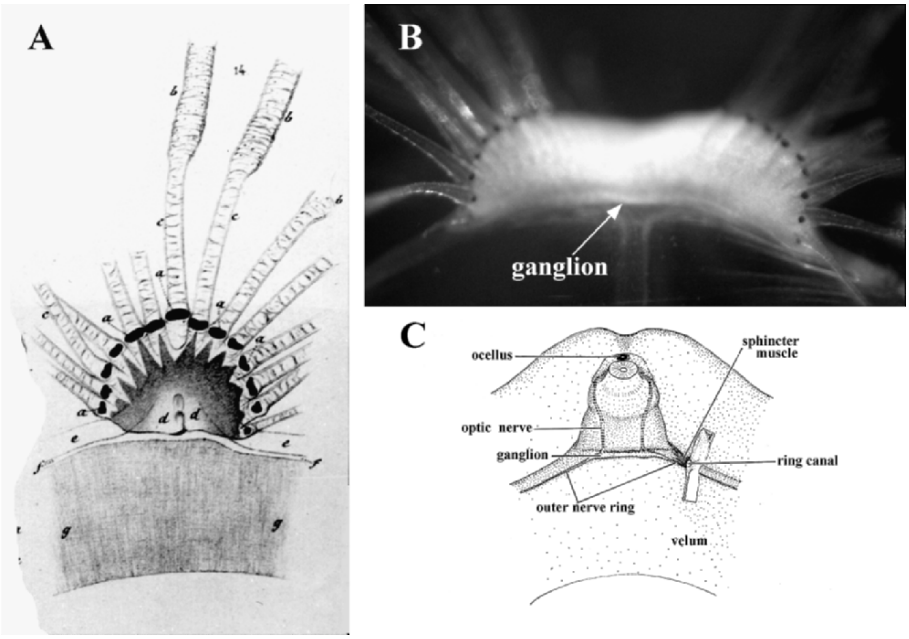

Figure 2. (A) Tentacle bulb of *Bougainvillea superciliaris*, seen from below the velum, showing a, ocelli; b, distal portion of tentacles; c, proximal portion of tentacles; d, ganglion; e, ring canal; f, nerve ring; g, velum (from Agassiz, 1850). (B) Photo of tentacle bulb of *B. principis*, viewed as in A, showing a structure tentatively identified as the ganglion. (C) drawing showing a similar view of the tentacle bulb of *Sarsia* (from Mackie, 1971).

the microscope''. All relevant observations were made on fresh material. The main components of the nervous system as described by Agassiz are (a) cords or strings of ovate cells running around the margin close to the circular gastrovascular canal, (b) a swelling of the cord (''a sort of ganglion'') at each of the four tentacle bases (*Sarsia* and *Bougainvillea*), (c) radial nerves running up the subumbrella alongside the radial canals, (d) extensions of the latter into peduncle and manubrium, and (e) lateral branches from the radial nerves that interlink to form an upper nerve ring that encircles the peduncle.

Methods

Specimens of *Bougainvillea principis* (Steenstrup) were obtained off the dock at the Friday Harbor Laboratories of the University of Washington. The species very closely resembles *B. superciliaris* (= *Hippocrene superciliaris* Agassiz), differing from it chiefly in its greater number of tentacles. Specimens were fixed in 4% formaldehyde in 0.1 M phosphate buffered saline (PBS), washed in PBS containing 0.3% Triton X-100 and 0.03% sodium azide, and labeled with either 1:200 rabbit anti-FMRFamide serum (Peninsula Laboratories, IHC 8755) or mouse anti-tubulin antibody (Amersham, N356). Preparations were viewed after labeling with the appropriate secondary antibodies using a Biorad MRC-600 laser scanning confocal microscope. A Nikon Coolpix 995 digital camera was used for photographing whole mount preparations.

Results

Nerve ring and ganglia

A nerve ring was observed in *Bougainvillea* in the exact location described by Agassiz (1850), and it appeared to swell up at each of the four tentacle bulbs (Figs 2A and B) as he describes. Without sectioning this region for transmission electron microscopy it would be difficult to be sure that the structure indicated is indeed a ganglion, but a similar structure exists in *Sarsia* (as Agassiz noted), and this has been sectioned and found to

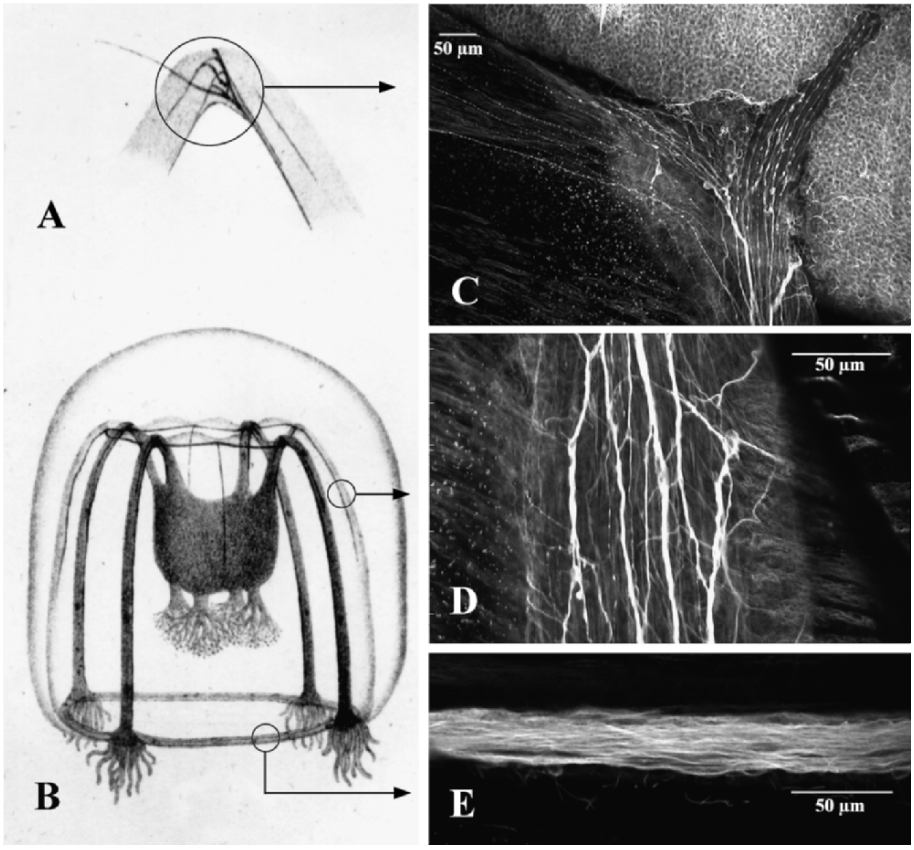

Figure 3. Drawings of *B. superciliaris* showing nervous structures (from Agassiz, 1850: Pl. III, Figs. 5 and 6). (A) "The plexuses under the arches of the radiating tubes". (B) Principle nervous structures in summary. C–E. Photomicrographs of the principle structures, as indicated by the arrows, from whole mounts of *B. principis* immunolabeled with anti-tubulin antibody. "(C) apex of radial nerve plexus where nerves branch off laterally (left) forming the upper nerve ring. (D) Enlarged view of radial nerve plexus. (E) Outer nerve ring.

show the structural characteristics of a ganglion, with a cortical layer of cell bodies surrounding an inner medullary region containing about 1000 axon profiles (Mackie, 1971, and Fig. 2C). The ganglion is continuous with the outer nerve ring. Anti-tubulin labeling of a marginal strip in *Bougainvillea* showed axons in the outer nerve ring (Fig. 3E).

Radial nerves

Nerve plexuses were observed in *Bougainvillea,* running up from the inner nerve ring and lying in the ectoderm. There were four such "radial nerves", each overlying one of the radial canals. The plexus showed tubulin immunoreactivity (Fig. 3D). A subset of axons within the plexus also showed FMRFamide immunoreactivity (not figured).

Extensions of the radial nerves into the peduncle and manubrium

The majority of the axons comprising the radial nerves, including the entire FMRFamide-immunoreactive component, were found to continue into the peduncle (Fig. 3C), where they merge with a general ectodermal nerve plexus in the manubrium.

Upper nerve ring

Groups of tubulin-immunoreactive axons were observed to diverge laterally from the tops of the radial nerves (Fig. 3C), where they formed an arch

across the top of each quadrant of the subumbrellar muscle layer, joining with similar branches from adjacent radial strands. Together, these four arches can be considered to comprise an upper nerve ring. A similar structure has been observed in *Stomotoca atra* Agassiz (Mackie & Singla, 1975) and in *Polyorchis penicillatus* (Lin et al., 2001). One feature shown in the drawings could not be confirmed; i.e. the inter-radial nerve tracts running down from the upper nerve ring.

Discussion

There can be little doubt that Agassiz saw marginal nerve rings in several species and, in the case of *Bougainvillea* and *Sarsia*, he also saw the swellings now known as tentacular ganglia. These centers presumably represent points where large numbers of nerves from the tentacles and ocelli enter the outer nerve ring. In *Polyorchis*, a substantial subsystem of the nervous system (the "O" system) is associated with ocellar input; depolarizations of the "O" neurons in response to light modulate output of the swimming pacemakers (reviewed by Satterlie & Spencer, 1987). Ganglia and brains typically arise close to sense organs and function to integrate sensory input with motor output, and *Bougainvillea* is exceptionally well endowed with ocelli, having 11–15, sometimes as many as 22, at each tentacle bulb in the case of *B. superciliaris*.

Agassiz describes the cells comprising the nerve rings as "ovate" and figures them as such (1850: pl. III, Fig. 13) without showing their axons. Most of the axons in medusan nerve rings have diameters in the 0.5–1.0 μm range. It is doubtful if the lenses available in the 1840s could resolve much detail in this range (Bradbury, 1967) unless the axons could somehow be made to appear self-luminous by ingenious oblique lighting. Given the inherent optical limitations of his lenses and the fact that Agassiz was immersing his objective lens in sea water containing a living, moving animal, the failure to observe individual axons comprising the nerve rings is not surprising.

The radial nerves running up from the nerve ring certainly exist in the regions indicated by Agassiz, but rather than being single fibres as he shows them, they are actually plexuses containing a mixture of thick and thin strands, mostly elongated in the radial direction (Fig. 3D). We may assume Agassiz saw only the thicker strands (bundles of dozens of individual axons) and portrayed them as a single line for simplicity. In the vault of the subumbrellar cavity, the radial nerves do indeed branch as Agassiz described, a major group continuing on into the peduncle and manubrium and smaller groups diverging to left and right to form the inter-radial arches comprising the upper nerve ring. Here too, what we know to be plexuses (Fig. 3C, D) are simplified as single lines. An elegant study by Lin et al. (2001) on *Polyorchis* shows that the arches are part of the swimming motor neuron network and innervate the striated swimming muscles. While conduction in the muscle sheet is myoid, contractions are presumably initiated by nervous input from all sides, an adaptation that would increase synchrony.

The nerves shown by Agassiz descending interradially from the upper nerve ring were not seen in the present investigation, and nothing comparable has been reported for *Polyorchis* or *Stomotoca*. This then is one place where his account may be in error.

Perhaps what strikes the present-day reader of Agassiz's account most is its professionalism. Methods and results are described in great detail, conclusions are expressed cautiously, alternative explanations are considered, earlier literature is scrupulously cited, and great pains are taken to make the illustrations as realistic as possible. Swiss by birth, Agassiz had been trained in Zürich and Heidelberg, coming under the influence of leading figures like H.G. Bronn, F. Leuckart, T. Bischoff, and F. Tiedemann. Moving to Munich at the age of 20, where he obtained doctorates in Philosophy and Medicine, he worked on Brazilian fishes, publishing a monograph on his researches two years later, then on to Paris for work on fossil fishes backed by G. Cuvier and A. von Humboldt. His *Poissons Fossiles* (1843), along with his formulation of the Ice Age concept from extensive study of alpine glaciers and moraines (*Etude sur les glaciers,* 1840), established him as a major figure, trained to the highest European standards, and with a remarkable breadth of scientific interests. These skills, standards and interests he brought with him to America, and are well expressed in the

first major work he published in the U.S.A, the Memoir that is the subject of the present paper (Agassiz, 1850). The recognition he received in the land of his adoption is easy to understand given the prestige he brought with him from Europe, his charismatic personality, and the quality of the work he produced.

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Part II
Neurobiology

Introduction to the Symposium

The research presented in this two-day symposium attests not only to the coming of age of coelenterate neurobiology, but also to provocative and far-reaching questions posed by the unique organization of the coelenterate coordinating system. Molecular, biochemical, ultrastructural, immunocytochemical, and electrophysiological techniques have stimulated new questions, even as old ones have been resolved. They are forcing a renewed appreciation of the subtlety and precision of the coelenterate behavioral design.

The papers that follow bear witness to the growing consensus that coelenterates are exceedingly catholic in their coordinating mechanisms. Epithelial conduction, by means of electrically conducing gap junctions, and gas-mediated epithelial coordination of cnidae and myonemes, together with classical, neurotransmission and peptidergic modulation in the same nerves – and, importantly, the highly organized eyes of some forms – make it clear that the apparent simplicity of coelenterate neuro-effector systems is just that, an apparent simplicity. Micro-anatomical and chemical differentiation within a nervous system, inter-tissue communication between neurons and epithelia, between neurons and cnidocytes, and between epithelia and cnidocytes, reveal the surprising complexity of these once seemingly homogeneous coordinating systems.

The intricate and specific integration of sensory signals, the diverse array of receptors, ligands, conduction pathways, and effectors, and the molecular relationships to other species are forcing a revised assessment not only of the place of coelenterates in evolution, but also of their highly developed physiology. The research that follows presents a review of the state of coelenterate neurobiology that is at the same time satisfying and challenging.

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Biogenic amine receptors in the sea pansy: activity, molecular structure, and physiological significance

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Key words: Renilla koellikeri, biogenic amines, aminergic receptors, peristalsis, bioluminescence

Abstract

Although cnidarian nervous systems appear to make heavy use of neuropeptides, evidence of the presence of biogenic amine transmitter systems has also surfaced over the past two decades. Our and other laboratories have used several analytical methods to reveal the presence of catecholamines and indoleamines in cnidarians, some of which are traceable to specific subsets of neurons. Evidence was also provided of amine biosynthesis, and of neuronal catecholamine release. Adrenergic-like and serotonin-like receptors were pharmacologically characterized by bioassays and by radiobinding assays in the sea pansy *Renilla koellikeri*, and these receptors appear to mediate activities such as bioluminescence and peristaltic contractions. The search for their structural identity has led to the molecular cloning and characterization in the sea pansy of two new G protein-coupled receptors, Ren1 and Ren2, exhibiting homologies with aminergic receptors. In contrast to the receptors characterized in sea pansy membranes, none of the two cloned receptors responded to conventional amines when expressed in mammalian cell lines. Ren1 displayed constitutive cyclic AMP-generating activity when expressed in mammalian cells. These findings illustrate the challenges posed by attempting to reconcile physiological and molecular data in the quest for functional relevance of cnidarian transmitter systems.

Introduction

Understanding how neurotransmission works in the earliest manifestations of neural networks and how it evolved from there is a worthwhile pursuit of comparative neurobiology. The basal phylogenetic position of Cnidaria provides a valuable opportunity to examine the extent, and manner in which use was made of chemical neurotransmission as the first nervous systems were emerging.

Investigations of chemical neurotransmission in Cnidaria have progressed considerably in the past two decades. The extraction and identification of numerous neuropeptides from a variety of cnidarian species, and their immunohistochemical visualization in neurons throughout cnidarian nervous systems, have largely contributed to the current status of the field (Grimmelikhuijzen et al., 2002). Among the smaller, conventional transmitter substances, amino acids such as glutamic acid, glycine, γ-aminobutyric acid, and taurine were found to be present in cnidarian neurons and to be active in feeding and nematocyst discharge modulation, possibly through specific receptors in some cases (Bellis et al., 1991; Pierobon et al., 1995, 2001).

A major roadblock to progress in this field is the fragmented body of data on neurotransmitters disseminated among various cnidarian species,

thus making it difficult to bring together all criteria needed to establish the involvement of a neurotransmitter system in one specific neural pathway of a single species. Simply stated, these criteria are: (1) the transmitter substance is synthesized in, and released by, the stimulated neuron, (2) there exist mechanisms (presynaptic reuptake or enzymatic degradation) to inactivate excess released transmitter, (3) there exist 'postsynaptic' receptor signalling mechanisms for the suspected transmitter, and (4) the suspected transmitter, applied exogenously, mimics the action of the transmitter released by neuronal stimulation. Challenges to meet the last two criteria are (1) the lack of organs in the great majority of cnidarians, thus making it difficult to relate episodes of neuronal release to an action on a circumscribed functional unit, and (2) the pitfalls of identifying cnidarian transmitter receptors on the basis of the only existing classification schemes, constructed from mammalian complements of ligands and receptors.

This review presents evidence collected in the last 10 years of the presence and functional involvement of biogenic amine receptors in a single species, the sea pansy *Renilla koellikeri* (Anthozoa, Octocorallia). We will begin by summarising data that show to what extent criteria other than postsynaptic (receptor) mechanisms are met toward establishing biogenic amines as neurotransmitters in the sea pansy. Monoamines as putative neurotransmitters in Cnidaria were earlier reviewed by Anctil (1989b).

Biogenic amines in the sea pansy

The presence of catecholamines (De Waele et al., 1987) and of their inactivating (reuptake) mechanisms (Anctil et al., 1984) were first investigated in the sea pansy after actions of exogenous catecholamines (adrenaline and noradrenaline) on bioluminescence and muscle contraction were reported (Anctil et al., 1982). The three conventional transmitters dopamine, noradrenaline, and adrenaline were thus identified in sea pansy extracts using two different analytical methods, radioenzymatic assays and high-performance liquid chromatography (HPLC). Evidence of transmitter-inactivating mechanisms was obtained from two sources: both catecholamine reuptake and monoamine oxidase inhibitors potentiated bioluminescent responses to adrenaline (Anctil et al., 1982), and autoradiography showed high-affinity uptake of exogenous tritiated catecholamines in neurons and in non-neuronal cells (Anctil et al., 1987). Later, a more comprehensive HPLC analysis quantified the content not only of the three main catecholamines, but also of their precursors and degradation products (Pani & Anctil, 1994a). In addition, serotonin (5-HT) and related indole products were detected. HPLC evidence of enzymatic conversion of biogenic amines was also obtained (Pani & Anctil, 1994b).

Because the sea pansy has no distinctive nerve-rich tissue, the association of biogenic amine content with neurons was investigated by immunohistochemistry. Numerous serotonin-immunoreactive sensory neurons were thus identified in the ectoderm of polyps and colonial tissue, and in some mesogleal and endodermal neurons (Umbriaco et al., 1990). Another indoleamine derived from serotonin, melatonin, was reported in neurons morphologically similar to the serotonin-containing neurons (Mechawar & Anctil, 1997). The distribution of indoleaminergic neurons was consistent with the reported modulatory effects of exogenous serotonin and melatonin on peristaltic contractions of the sea pansy (Anctil, 1989a; Anctil et al., 1991). In spite of the wide range of catecholamines detected in the sea pansy, only noradrenaline-immunoreactive neurons have been identified so far, largely in the mesogleal nerve net (Pani et al., 1995). This was confirmed by immunohistochemical evidence of dopamine-β-hydroxylase, the noradrenaline-synthesizing enzyme, in neurons and associated amoebocytes of the mesogleal nerve net as well as in some ectodermal and endodermal neurons (Anctil et al., 2002).

Evidence of neuronal release of catecholamines in the sea pansy is twofold. First, in sea pansy preparations pre-loaded with tritiated noradrenaline, transient, calcium-dependent outflow release of labelled noradrenaline was evoked by field electrical stimulation that also elicited nerve net-mediated bioluminescence (Pani et al., 1995). The second, more direct evidence came from amperometric detection of catecholamine release by single neurons in culture (Gillis & Anctil, 2001). This study allowed the detection in real time of exocytotic events, revealing two populations of catecholaminergic neurons distinguished by their

pattern of secretion (evoked versus spontaneous). Unfortunately, the active catecholamine could not be identified in the latter study.

Pharmacological evidence of aminergic receptors

As shown above, there is extensive evidence that criteria for biogenic amines playing a neurotransmitter role are largely met in the sea pansy, at least on the 'presynaptic' side. What is the evidence on the 'postsynaptic' side? We first looked for receptors associated with the adrenergic control of bioluminescence. Using two radioligand binding assays on sea pansy membrane extracts, two distinct classes of adrenergic-like binding sites were identified (Awad & Anctil, 1993a), one present in the polyps and associated with bioluminescent tissue (site 1) and the other present in the colonial mass (site 2). Site 1 exhibited a pharmacological profile similar to mammalian β2-adrenoceptors and site 2 to β1-adrenoceptors, but neither site fit snugly in the mammalian classification scheme, as would be expected from the wide phylogenetic gap separating the two phyla. While the pharmacological profile of bioluminescence correlated significantly with that of site 1, no physiological activity could be clearly associated with site 2 (Awad & Anctil, 1993a). The association of site 1 with bioluminescent tissue was confirmed by combined radioligand autoradiography and in situ hybridisation using an oligonucleotide probe complementary to human β2-adrenergic receptor DNA (Awad & Anctil, 1994).

Interaction of a β-adrenergic agonist with its binding site on the cell surface usually stimulates a GTP-binding protein (G protein) which in turn activates the membrane-bound enzyme adenylate cyclase that catalyses the formation of the second messenger cyclic AMP from ATP. This was demonstrated in the sea pansy, first by showing that GTP and its analogues stimulate adenylate cyclase activity from membrane fractions, then by showing a close correlation between the pharmacological profile of adrenergic agonist-induced adenylate cyclase activity and that of binding at sites 1 and 2 (Awad & Anctil, 1993b). The stimulatory effect of cholera toxin on adenylate cyclase activity established that a stimulatory G protein (Gs) was involved (Awad & Anctil, 1993b). This study also revealed that other aminergic binding sites distinct from sites 1 and 2 (especially a serotonin-binding site) were able to activate adenylate cyclase.

Experiments showing that serotonin potentiates peristaltic contractions through the mediation of cyclic AMP in the sea pansy (Anctil, 1989a) led to the characterization by a radioligand binding assay of one type of serotonin receptor (Hajj-Ali & Anctil, 1997). This receptor proved to be even less pharmacologically classifiable than the adrenergic sites due to the relatively poor affinity of the majority of tested serotonergic antagonists for it. The sea pansy receptor also stands apart from all known invertebrate serotonin receptors by its complete inability to bind LSD. This unorthodox profile prompted us to suggest that the sea pansy receptor predates the evolutionary radiation of the multiple classes of serotonin receptors (Hajj-Ali & Anctil, 1997). On the other hand, this receptor conserved its coupling to a G protein as demonstrated by the effect of a GTP analogue on binding (Hajj-Ali & Anctil, 1997), a result consistent with the strong stimulatory effect of serotonin on adenylate cyclase activity and cyclic AMP production (Awad & Anctil, 1993b).

It is interesting that the pharmacological profile of the sea pansy receptor resembles best that of the 5-HT_{1P} serotonin receptor associated with the myenteric plexus of the mammalian gut (Branchek et al., 1984), where serotonin also modulates peristalsis. The possible role of the sea pansy serotonin receptor in peristalsis is supported by the finding that serotonin antagonists with a piperazine-like moiety are the most potent in blocking the modulatory effect of serotonin on sea pansy peristalsis (Anctil, 1989a) as well as in competing with tritiated serotonin for binding sites (Hajj-Ali & Anctil, 1997).

Molecular cloning of aminergic receptors

The pharmacological and functional studies reviewed in the previous section suggest the presence of multiple G protein-coupled biogenic amine receptors in the sea pansy. In order to eventually understand how the structure of these receptors and their conformation in the cell membrane may determine their ligand-binding and G protein-coupling activities, it is imperative to clone them, analyse their sequence, and express them in

suitable cell systems for functional assays. In addition, cloning such receptors in a cnidarian provides a unique opportunity to compare their molecular structure with that of higher metazoans and gain some understanding of the evolution of G protein-coupled receptors (GPCRs), which represent the largest family of transmembrane receptors responsible for the transduction of a wide range of extracellular signals. Therefore, we undertook a search for such receptors in the sea pansy by molecular cloning, and two putative monoaminergic receptors, Ren1 and Ren2, were cloned.

For the molecular cloning of the cDNA of Ren1 we obtained a first clone by PCR amplification of cDNA from polyps with degenerate oligonucleotide primers corresponding to the highly conserved sequence for transmembrane region VI and VII of biogenic amine receptors. In order to obtain the 3′ end region of the receptor cDNA, a 3′ RACE approach was adopted. The clone that completes the sequence is a PCR product performed on a sea pansy polyp cDNA library using a pair of specific primers deduced from the receptor cDNA and the phage arm. The whole coding region was amplified from cDNA with the enzyme *pfu* to confirm the sequence. The open reading frame of Ren1 cDNA encodes for a protein of 344 amino acids which contains the typical seven hydrophobic domains of GPCRs presumed to be embedded in the cell membrane (Bouchard et al., 2003). In addition, amino acid residues and motifs conserved in most GPCRs are present in Ren1.

When the amino acid sequence of Ren1 was compared with other known sequences, the highest homologies (near 45%) were found with vertebrate biogenic amine receptors, especially dopaminergic and adrenergic receptors (Bouchard et al., 2003). Consistent with this finding, several amino acid residues typically found among receptors that are activated by amine ligands are encountered in Ren1 (Bouchard et al., 2003). While Ren1 is clearly related to amine receptors, a dendogram analysis by Bouchard et al. (2003) of over 50 monoamine GPCRs showed that Ren1 could not be associated with any of the known subclasses of aminergic receptors.

In order to functionally characterize Ren1, it was expressed transiently and stably in HEK-293 cells, and transiently in COS-7 and LTK cells (Bouchard et al., 2003). As an affiliation with aminergic receptors emerged from sequence analysis, ligand screening of the expressed receptor was performed primarily with natural biogenic amines, their precursors, and metabolites. None of the tested ligands, including amines and other putative transmitter substances previously detected in the sea pansy, was able to induce a response even when using a variety of receptor signalling assays (CRE-SEAP, direct cyclic AMP, and calcium assays) and radioligand binding assays. Surprisingly, the cells expressing Ren1 displayed high basal levels of cyclic AMP compared with mock-transfected or untransfected controls (Bouchard et al., 2003). These data led us to conclude that Ren1 is at least provisionally an orphan receptor which is capable of constitutive activity involving a cyclic AMP second messenger pathway.

The cDNA of the Ren2 receptor was cloned using a strategy similar to that used for Ren1. As for Ren1, the deduced amino acid sequence from the reconstructed full-length cDNA of Ren2 revealed the seven putative transmembrane domains typical of GPCRs (Bouchard et al., 2000). The 323 amino acid polypeptide revealed up to 45% similarity with histaminergic and β2-adrenergic receptors. As Ren1, Ren2 shares key structural features with GPCRs, but differs from Ren1 by substituting the conserved DRY motif with a DRC pattern at the cytoplasmic side of the third transmembrane domain, and by missing the conserved cysteine residues usually found in the second extracellular loop and in the C-terminal tail (Bouchard et al., 2000). Several key amino acid residues conservatively associated with aminergic receptors are also present in transmembrane domains of Ren2. Although biogenic amines appeared to be the obvious choice for ligand screening of HEK-293 cells transfected with Ren2, no response was obtained with the cyclic AMP assays previously used for Ren1 (unpublished). Consequently, both Ren1 and Ren2 appear to be orphan receptors.

Conclusions

The pharmacological profiles of all three putative biogenic amine receptors detected by radiobinding studies of sea pansy membranes reveal sensitivity to conventional amine transmitters but substantial departure from the known classes of aminergic

receptors in higher invertebrates and vertebrates. The structural information in sequences of two GPCR receptors cloned from the sea pansy points to a high homology with aminergic receptors in terms of key amino acid residues, but also to structural divergences that preclude their affiliation with specific classes of aminergic receptors. Their ligands were not identified, thus implying that they are not related structurally or functionally with the putative receptors pharmacologically characterized from sea pansy membranes. This raises the possibility that the sea pansy possesses several aminergic-like GPCRs with characteristics (structural or pharmacological divergence from all known aminergic receptors, orphan status, one case of constitutive activity) that suggest a long history of receptor sequence diversification in the course of metazoan evolution.

Several unresolved issues remain to be addressed. One is the disconnection between aminergic receptors that appear to have specific ligands but no structural identity when examined in sea pansy membranes, and aminergic receptors that have a structural identity but no ligand when cloned. In order to obtain a complete picture of these receptors, from molecular structure through ligand to physiological function, more aminergic receptors will have to be cloned and more effort to screen for unusual amine ligands in the sea pansy will need to be made. The issue of orphan receptors may not be resolved simply by ligand screening, and it may require the development of a cnidarian cell line for expression studies of cloned receptors to obviate the possibility that mammalian cells are ill-suited environments for expressed cnidarian receptors. When all these issues are addressed and orphan and constitutively active receptors still remain, then the possibility should be addressed that competent receptor proteins without access to conformationally adequate ligands existed in these early metazoans. Some of these receptors, like Ren1, may have physiologically relevant constitutive activity, possibly involved in the long-term maintenance of cellular functions.

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Chemical anatomy of hydra nervous system using antibodies against hydra neuropeptides: a review

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Abstract

Polyclonal antibodies against hydra neuropeptides allow us to visualize the hydra nerve net. Chemical anatomy of the hydra nerve net was archived by means of immunocytochemistry with various antibodies to hydra neuropeptides. Our goal is to describe the hydra nerve net both qualitatively and quantitatively. The present chemical anatomy results indicate (1) differences in peptide expression between ganglion cells and sensory cells, (2) large differences in neuropeptide expression between ectodermal nerve cells and endodermal nerve cells, and (3) the usefulness of quantitative chemical anatomy to analyze the entire nervous system of hydra.

Introduction

Hydra has a simple nervous system consisting of a nerve net that extends throughout the body (Hadži, 1909; Burnett & Diehl, 1964; Lentz & Barrnett, 1965; Lentz, 1968). Nerve cells are interspersed among the epithelial cells of both layers. No large concentrations of neurons such as ganglia are observed (Lentz & Barrnett, 1965; Lentz, 1968; Koizumi et al., 1992). The nerve net contains two types of cells, ganglion cells and sensory cells (Davis et al., 1968; Koizumi et al., 1988; Grimmelikhuijzen & Westfall, 1995). Ganglion cells lie close to the muscle processes at the basal ends of the epithelial cells. Sensory cells have elongated cell bodies that extend from the level of the muscle processes in an apical direction, and an elaborate ciliary cone at the apical end of the cell body (Westfall, 1973; Westfall & Kinammon, 1978; Bode et al., 1988; Koizumi et al., 1988; Grimmelikhuijzen & Westfall, 1995).

Immunocytochemistry using neuropeptide antisera and monoclonal antibodies specific to hydra neurons on whole mounts has made it feasible to study the nerve net (Grimmelikhuijzen, 1985; Dunne et al., 1985; Koizumi & Bode, 1986, 1991; Bode et al., 1988; Koizumi et al., 1988; Minobe et al., 1995). These studies have shown that the nerve net contains numerous subsets of neurons and that the spatial distributions are highly position-specific. Several subsets defined by monoclonal antibodies have been noted (Dunne et al., 1985; Koizumi et al., 1988; Yaross et al., 1986). The regional distribution of each subset tends to be constant under the active developmental dynamics of nerve cells in an adult (Koizumi et al., 1988; Bode et al., 1988; Bode, 1992).

Recently, we have initiated a systematic effort to isolate peptide signaling molecules from hydra (Takahashi et al., 1997; Bosch & Fujisawa, 2001; Koizumi, 2002; Fujisawa, 2003). Takahashi et al. (1997) developed a novel procedure for systematically isolating peptide signal molecules. Peptides were extracted from large numbers of hydra and purified to homogeneity using high-performance

liquid chromatography (HPLC) without any biological assays. The isolated peptides were subjected to structural analysis using automated amino-acid analysis and then synthesized chemically, and the identity of synthetic peptides with native peptides was confirmed using HPLC. The synthetic peptides were then subjected to a series of biological tests to examine their functions in hydra. Using this approach, a number of peptides have been identified that regulate development in addition to neuropeptides controlling synaptic transmission and muscle contraction (Takahashi et al., 1997, 2000, 2003; Yum et al., 1998; Grens et al., 1999; Bosch & Fujisawa, 2001; Harafuji et al., 2001; Koizumi, 2002; Fujisawa, 2003; Morishita et al., 2003).

We produced polyclonal antibodies against all of the peptide molecules identified in our hydra peptide project (large scale screening of peptide signal molecules in hydra). Immunostaining using these antibodies shows that half of the peptides are neuropeptides, while half are epitheliopeptides which localize in epithelial cells. Table 1 is the list of hydra neuropeptides whose structures and functions are identified (Moosler et al., 1996; Takahashi et al., 1997, 2000, 2003; Yum et al., 1998; Morishita et al., 2003). We now have new tools,

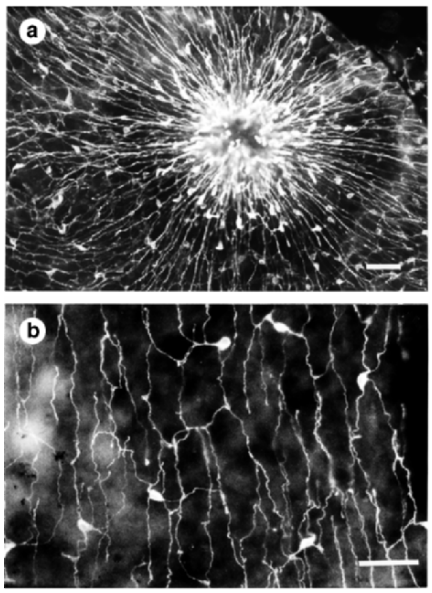

Figure 1. Nerve net in hydra, visualized immunocytochemically on the whole mount animal. Patterns of the nerve nets are different depending on the position. (a) RFamide-like immunoreactive ($RFamide^+$) nerve net in the hypostome. A view from above the hypostome shows the dark mouth region in the center surrounded by large number of epidermal sensory cells. (b) $RFamide^+$ nerve net of upper body column. Bars indicate 50 μm.

Table 1. Hydra neuropeptides whose structure and function are identified

Peptide	Structure	Function	Reference
RFamide			Moosler et al. (1996)
Hydra-RFamide I	pQWLGGRFamide	Myoactive	
Hydra-RFamide II	pQWFNGRFamide		
Hydra-RFamide III	KPHLRGRFamide		
Hydra-RFamide IV	HLRGRFamide		
GLWamide			Takahashi et al. (1997, 2003)
Hym-53	NPYPGLWamide	Muscle contraction	
Hym-54	GPMTGLWamide		
Hym-248	EPLPIGLWamide		
Hym-249	KPIPGLWamide		
Hym-331	GPPPGLWamide		
Hym-338	GPPhPGLWamide		
Hym-370	KPNAYKGKLPIGLWamide		
Hym-176	APFIFPGPKVamide	Muscle contraction of ectoderm	Yum et al. (1998)
Hym-355	FPQSFLPRGamide	Activation of the nerve differentiation	Takahashi et al. (2000)
Hym-357	KPAFLFKGYKPamide	Antagonism of Hym-176	
Arg-vasopressin	CYFQNCPRGamide	Hym-355	Morishita et al. (2003)

neuropeptide antibodies, to visualize the hydra nerve net.

We studied the chemical anatomy of the nervous system using antisera against hydra neuropeptides (Table 1) (Koizumi et al., 2000; Goto et al., 2000; Koizumi & Sato, 2001). The present chemical anatomy shows (1) differences of peptide expression between ganglion cells and sensory cells, (2) large differences in neuropeptide expression between ectodermal nerve cells and endodermal nerve cells, and (3) the usefulness of quantitative chemical anatomy to analyze the entire nervous system of hydra.

Qualitative chemical anatomy

We made a detailed qualitative chart of the chemical anatomy showing the spatial distribution of each neuronal subset and the overlapping and non-overlapping of subsets between each other. It shows

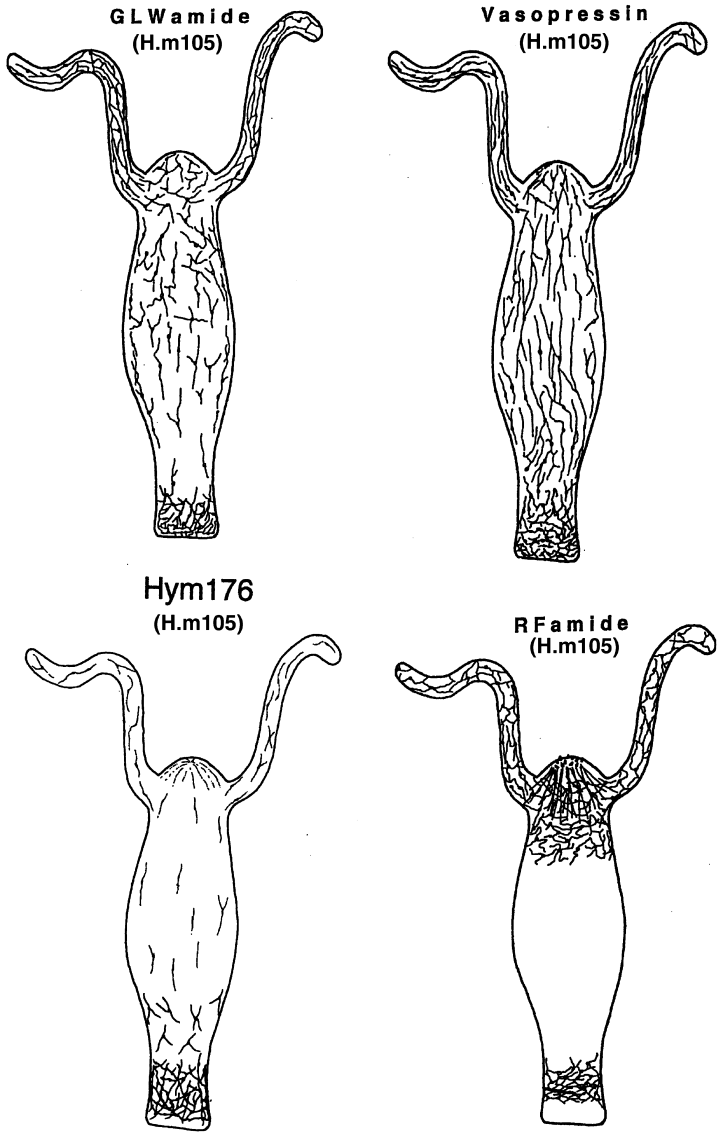

Figure 2. Illustration of spatial distribution patterns of immunoreactive neurons by various neuropeptide antisera. Nerve nets of *Hydra magnipapillata* visualized by antibodies against GLWamide, vasopressin, Hym-176, and RFamide, are illustrated.

Table 2. Immunostaining of sensory cells and ganglion cells in each region of hydra body

Antibody	Hypostome		Tentacle		Body column GC	Peduncle GC	Basal disk		Nerve ring
	SC	GC	SC	GC			SC	GC	
RF amide Ab	++	+	–	+	–	++	–	–	++
GLW amide Ab	–	+	–	+	+	+	–	+	+
Hym176 Ab	–	+	–	+	+	+++	–	+/–	–
Hym355 Ab	–	+	–	+	+	+	–	+	+
Vas. Ab	–	+	–	+	+	+	++	+	–

The data are shown for *Hydra oligactis*.
SC: sensory cell, GC: ganglion cell, Vas.: vasopressin.
+++: highly bright staining, ++: bright staining, +: staining, +/–: dimmer staining, –: negative staining.

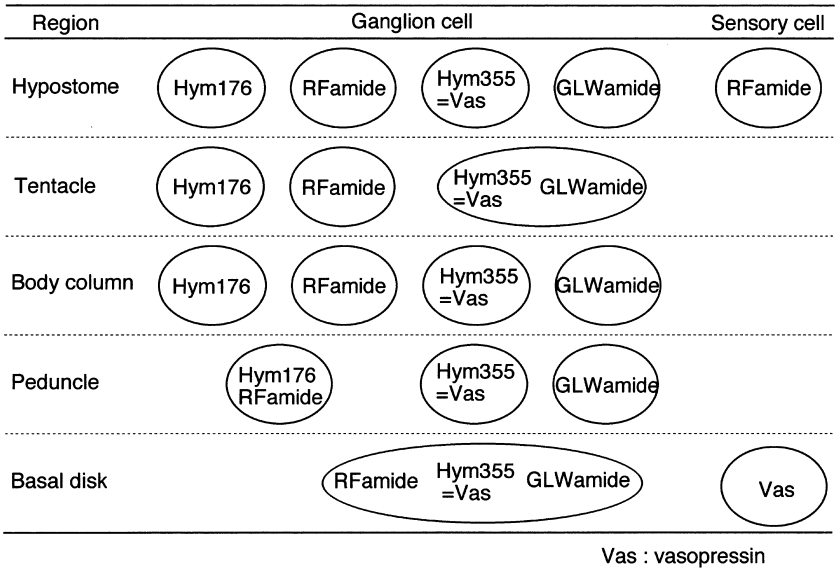

Figure 3. Diagram of neural subsets in each body region of *Hydra magnipapillata*. Overlapping or non-overlapping between subsets of ganglion cells determined by double staining with peptide antisera are shown. Non-overlapping sets indicate separate populations. This diagram shows that ganglion cells are a heterogeneous population, while sensory cells are a homogeneous population. 'Hym355 = Vas' indicates a vasopressin-like immunoreactive subset is identical to the Hym355-like immunoreactive subset (Morishita et al., 2003).

that ganglion cells are heterogeneous populations, whereas sensory cells are homogeneous populations. Ganglion cells contain many subsets, each of which expresses a different neuropeptide.

Whole mount immunostaining of hydra is an effective technique to demonstrate the three-dimensional structure of a major portion of the animal's nervous system (Fig. 1). The staining patterns of nerve nets were different depending upon which neuropeptide antiserum was used, as illustrated in Figure 2 (Grimmelikhuijzen, 1985; Koizumi & Bode, 1991; Yum et al., 1998; Takahashi et al., 2000, 2003). Table 2 is a summary of the immunostaining of sensory and ganglion cells in each region by various neuropeptide antibodies. This table indicates remarkable differences between them. Sensory cells expressed only one or no neuropeptides in each body region, while ganglion cells expressed many kinds of neuropeptides. That raises the question of whether or not (1) each ganglion cell expresses all of the neuropeptides, or, alternatively, (2) each ganglion cell expresses a single kind of neuropeptide. And do ganglion cells contain many different subsets?

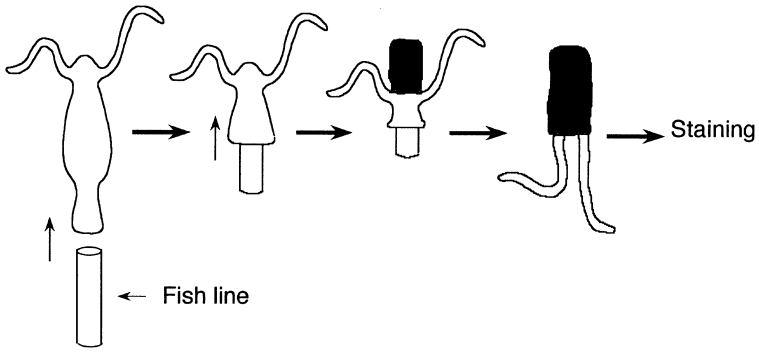

Figure 4. Method of producing inside-out hydra in order to examine the immunostaining of the endodermal layer. The method is very similar to Trembley's '*turning polyps inside out*' in his 1744 book (Lenhoff and Lenhoff, 1986:154) with the exception that he used a boar's hair while I used a fishing line. I pushed the fishing line against the foot of the animal so that the foot, followed by the rest of the hydra, eventually emerged through the mouth. The hydra was anesthetized with urethane soon after turning inside-out, and then was immunostained.

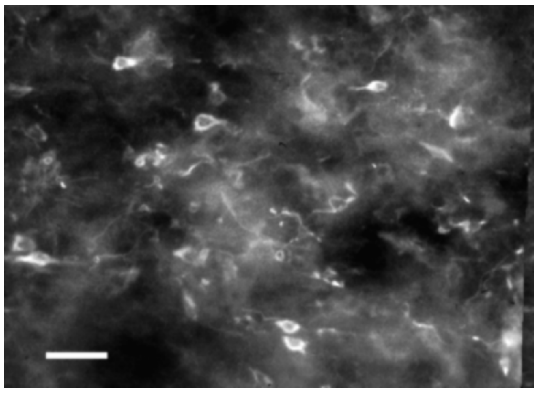

Figure 5. Neurons in hydra endoderm visualized by α-tubulin antibody. The bar indicates 50 μm.

To answer these questions, we did double staining to determine whether two neuropeptides are expressed in the same or different ganglion cells (Koizumi et al., 1992). In an example of RFamide/GLWamide double staining, we made RFamide antiserum using guinea pigs and GLWamide antiserum using rabbits. With specific second antibodies, RFamide-like immunoreactivity was visualized with green fluorescence of Fluorescein, and GLWamide showed red fluorescence with Texas Red. These double staining experiments using various combinations of two neuropeptides showed each ganglion cell expressed no more than one or a few neuropeptides (Fig. 3).

The diagram of subsets (Fig. 3) indicates that ganglion cells are a heterogeneous population, whereas sensory cells are a homogeneous population in each region of the hydra body (Koizumi et al., 2000). The biological significance of the remarkable differences between ganglion cells and

Table 3. Immunoreactivity of ectodermal and endodermal nerve net in hydra

Antibody	*Hydra magnipapillata*		*Hydra oligactis*	
	Ectoderm	Endoderm	Ectoderm	Endoderm
α-tubulin (Ms)Ab	++	++	+	++
Hym355 (Rb)Ab	+	–	++	–
Hym176 (Rb)Ab	++	–	++	–
GLWamide (Rb)Ab	++	–	++	–
Hym357 (Rb)Ab	+	–	+	–
RFamide (Rb)Ab	++	–	++	–

(Ms): mouse antibody, (Rb): rabbit antibody.
Legends to the table are the same to Table 2.

sensory cells is not understood so far, but should be considered from a phylogenetic viewpoint.

Endodermal nerve net

The presence of nerve cells of hydra has been demonstrated in the endoderm as well as in the ectodermal nerve net (Lentz, 1968; Davis, 1972; Epp & Tardent, 1978; Westfall et al., 1991; Murate et al., 1996). However, the nerve nets visualized by neuropeptide antisera as shown in Figures 1 and 2 are all ectodermal nerve nets. What about the endodermal nerve net? To examine this question in detail, we made inside-out hydras (Fig. 4) and visualized the endodermal nerve net by immunocytochemistry. Using a monoclonal antibody to α-tubulin, we could see endodermal nerve cells (Fig. 5). However, we never saw endodermal nerve cells using neuropeptide antibodies. As shown in Table 3, neuropeptide antibodies show only an ectodermal nerve net. Hence these experiments reveal a remarkable difference between the ectodermal and endodermal nervous systems in regard to neuropeptide expression (Goto et al., 2000).

Quantitative chemical anatomy

We made a detailed quantitative anatomical chart. In each region of hydra we listed the number of neurons of each subset and estimated the fraction of each subset among the total number of nerve cells. The total number of nerve cells was obtained by the maceration of hydra tissue of each region (Bode et al., 1973; David, 1973; Bode, 1996). These calculations of fractions seem to be a useful measure to an analysis of the entire nervous system of hydra (Koizumi & Sato, 2001).

Standard hydra species have about 6700 nerve cells (Bode, 1996). We are interested in what fraction of the nerve net can be visualized by immunostaining. Is it only the tip of the iceberg of the total number of neurons? Or is it a large fraction? Fractions of each subset in each region ranged widely from less than 10% to more than 30%. As to the actual fraction of neurons we could visualize throughout the body, we calculated that more than 30% of the nerve cells were visualized by immunostaining (Koizumi & Sato, 2001).

Conclusion

The present work provides a description of the hydra nerve net both qualitatively and quantitatively. This information is encouraging, because it suggests that a complete description of the entire nerve net will be possible by immunostaining using neuropeptide antibodies. Furthermore, it will generate fundamental data for developmental, neurobiological, and neuroethological studies on this simple nervous system.

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Neuropeptides and photic behavior in Cnidaria

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Key words: phototaxis, neuropeptide function, RFamide neuropeptides, LWamide neuropeptides, *Hydractinia*

Abstract

Peptides of the RFamide family occur in neurosecretory cells of all nervous systems of Cnidaria so far studied. Photoreceptive organs – if evolved in a cnidarian species – are always associated with neural cells showing RFamide immunoreactivity. Experimental evidence for the function of RFamides and other neuropeptides in nervous systems and photoreceptive organs is, however, scarce or lacking. RFamide and LWamide immunoreactivity were surveyed in photoreceptive organs of the hydromedusa *Cladonema radiatum*, in rhopalia of the scyphozoan *Aurelia aurita*, and in rhopalia of the cubomedusa *Tripedalia cystophora*. A possible function of neuropeptides in transmission of photic stimuli was assayed by analysing photic behavior in *Tripedalia*, which has highly developed eyes, and in the simply constructed planula of the hydroid *Hydractinia echinata*, in which the mode of light perception is unknown. In both species, light orientation was effectively prevented by RFamides administered to the animals in micromolar concentration. In contrast, among four other neuropeptides occurring in the larva of *Hydractinia*, only one interfered with phototaxis and then only at 10× higher concentrations. Planulae depleted of bioactive peptideamides also lost phototaxis while still locomotorily active. The results support the hypothesis that one possible function of RFamides in Cnidaria is to transmit photic stimuli to epitheliomuscular targets.

Introduction

Cnidaria are regarded to be the most primitive metazoa having a nervous system. Many of the neural cells are sites of peptide synthesis and a great variety of cnidarian neuropeptides is known (Schaller & Bodenmüller, 1981; Grimmelikhuijzen et al., 1989, 1994, 2002; Leitz et al., 1994; Gajewski et al., 1996; Takahashi et al., 1997, 2000). While much information is available on the distribution of neuropeptides in the nervous system, less is known about the function(s) of an individual peptide at the site of release. This study addresses the question of whether peptides of the RFamide type and other neuropeptides are involved in light-directed locomotion. In general, neuropeptides have been attributed a role as neurotransmitters or neurohormones. Sea anemone neuropeptides act as excitatory or inhibitory myomodulators in isolated muscle as well as in solitary anthozoan polyps (McFarlane et al., 1987, 1991, 1992). There is a growing body of evidence that in addition to their physiological functions, peptides are important effectors of developmental control (Schaller & Bodenmüller, 1981; Leitz et al., 1994; Hoffmeister, 1996; Gajewski et al., 1996; Lohmann & Bosch, 2000; Takahashi et al., 2000; Plickert et al., 2003).

Information on the cellular localization of neuropeptides has been obtained from immunocytochemical studies. Most is known about the distribution of peptides reacting with antibodies to Arg-Phe-amides (reviewed by Grimmelikhuijzen et al., 2002). RFamide immunoreactivity was observed in all cnidarian species so far studied. Peptides of the RFamide family occur in neurons associated with photoreceptive organs in Cnidaria.

The neural network termed 'O' system (Spencer & Arkett, 1984) of the outer nerve-ring projects via the ocellar nerves to the ocelli of the medusa *Polyorchis penicillatus*. Since the ocellar nerve was found to be also immunoreactive to anti-RFamide antibody, Grimmelikhuijzen & Spencer (1984) argued that input of photic signaling into the bell-margin ganglia could occur via neuropeptide-containing nerves. This view was confirmed by Lin et al. (2001). Also, ocelli-bearing rhopalia of cubomedusae and scyphozoans contain RFamide immunoreactive neurons in conspicuously close association with sense organs such as ocelli and statocysts (Martin, 2002, 2004), supporting the idea that RFamides may play a role in conveying photic stimuli to the nervous system. RFamides occur, however, also in species or life stages of cnidarians that do not have ocelli or known structures of photoreception. In four orders of hydromedusae not possessing ocelli, FMRFamide immunoreactivity was detected in a generally very similar distribution including in immunoreactive cells of tentacles, in the manubrium, in the bell margin, and associated with smooth muscle of the radial channels (Coe, unpublished). All polyp stages and planulae of Cnidaria so far studied contain cells that are either immunoreactive to anti-RFamide antibody or express the gene(s) encoding the preproproteins of RFamides (for review see Plickert, 1989; Grimmelikhuijzen et al., 2002; Plickert et al., 2003). It is remarkable that even Cnidaria lacking known organs of photoreception show photosensitivity. Extraocularly perceived light allows them to orient with respect to a light source; it coordinates diurnal migrations in pelagic species or triggers spawning at the onset of daylight or night.

In this study, we investigated the role of neuropeptides in photo-orientation of planulae of *Hydractinia echinata* as an example of a very simply structured cnidarian. For comparison, young medusae of the cubozoan *Tripedalia cystophora* were studied to represent a cnidarian species with highly developed ocelli that bear retinae and lenses. Two lines of experimentation were pursued. First, peptides that occur in planulae were synthesized and added to planulae or to young medusae of *Tripedalia* in a light-orientation experiment. Second, we studied the behavior with emphasis on photic behavior of planulae in which functions of peptideamides were knocked out. This was achieved by preventing amidation from the onset of neuropeptide precursor gene expression in embryonic development (Plickert et al., 2003). Antibodies to RFamide and to LWamides were used to study the distribution of the respective peptides in several cnidarian species.

Material and methods

Animal culture

Colonies of *Hydractinia echinata* were maintained as previously described (Plickert, 1989) with minor modifications. Animals were kept at 15 °C, fed 5× a week with a mixed diet of 3-d-old brine shrimp larvae and ground mixed cod, shrimps, and shellfish. Embryos were reared at 18 °C. Polyps of *Tripedalia cystophora* were cultured at room temperature in artificial seawater and fed freshly hatched brine shrimp nauplii. Metamorphosis into medusae was stimulated by elevating the temperature to 28–30 °C. Ephyrae were obtained from strobilating polyps of *Aurelia aurita* maintained in artificial seawater at 20 °C.

Immunocytochemistry

Nerve cells expressing neuropeptides were detected as previously published (Plickert, 1989). In order to detect RFamide-like immunoreactivity, amidation-sensitive anti-RFamide antibody 1773 IIIp was used. Antibody was produced in New Zealand rabbits injected with Arg-Phe-amide coupled via carbodiimide to bovine thyroglubuline. For LWamide immunocytochemistry, amidation-sensitive antibody 1676 IIp was used (prepared by T. Leitz, as outlined in Gajewski et al., 1996).

Peptides

LWamide and RFamide immunoreactivity was studied in photoreceptive organs of the hydromedusa *Cladonema radiatum*, the ephyrae of *A. aurita,* and young medusae of *T. cystophora*. He-LWamide I, He-RNamide, He-Ryamide, and Pol-RFamide II were synthesized according to the published preproprotein sequences (Gajewski et al., 1996, 1998) and purified by preparative HPLC. He-LWamide

II was purchased from Bachem (Bubendorf, Switzerland). Peptides were dissolved in de-aerated double-distilled water at 10 mmol/l and kept as stock solutions at −20 °C. From this stock, aliquots were diluted in seawater to the final concentration.

Light-orientation apparatus and set up of experiments

Photo-orientation was analyzed in cuvettes 4 mm wide and 40 mm long, in a volume of 1 ml. Bottom and side walls of the apparatus were constructed from light-impermeable material; transparent front and rear windows of the cuvettes permitted directed illumination of animals. The experiment was placed in a back-light-shielded box in a north-facing window. Twelve cuvettes assembled in one apparatus allowed synchronous light-exposure of controls and animals treated with peptides in various concentrations (Figs 1 and 2). Either 20 young medusae of *T. cystophora* or 150 planulae of *H. echinata* were placed in the central third of the cuvette (Fig. 1A). The outcome was analyzed 16–20 h later (natural light regime including night and morning of the following day).

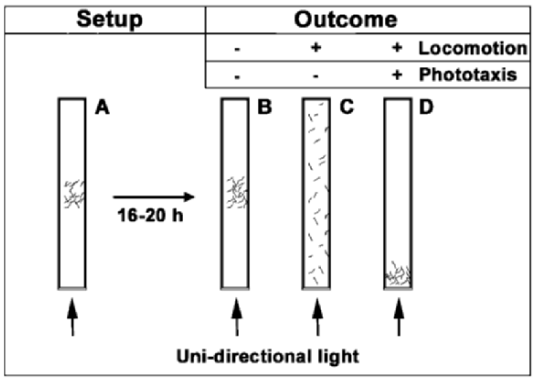

Figure 1. Behavior of *Hydractinia* planulae or *Tripedalia* medusae in response to unidirectional light. (A) Either 20 *T. cystophora* medusae or 150 *H. echinata* planulae were placed in the middle third of a cuvette 4 mm wide and 40 mm long. Animals were incubated in 1 ml of seawater or in seawater supplemented with peptides, then exposed to unidirectional daylight. (B) Animals stayed in the central area if locomotorily inactive. (C) They dispersed over the entire cuvette if locomotorily active but photo-actively neutral. (D) If attracted by light, animals accumulated at the light-exposed window of the cuvette (see also Fig. 2).

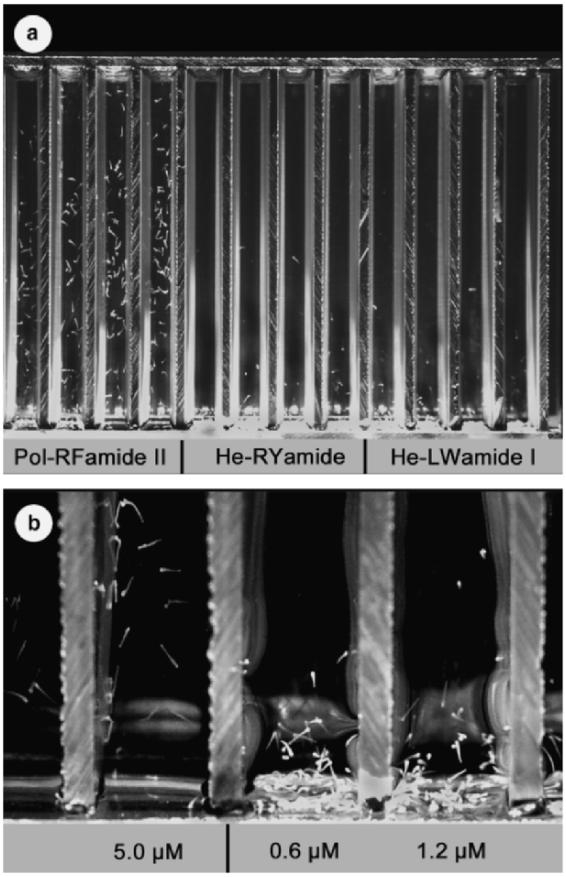

Figure 2. Effect of neuropeptides on photic behavior of *Hydractinia echinata* larvae. Approximately 150 planulae were placed in the central area of each of 12 cuvettes. The neuropeptides synthetic Pol-RFamide II, He-Ryamide, or He-LWamide I were added in a final concentration of 0.6, 1.2, 2.5, or 5.0 μM (from left to right). (a) Twenty hours later, larvae are dispersed over most of the area in cuvettes containing RFamide peptide (the same distribution as observed when larvae are kept in seawater in total darkness). Almost all larvae treated with other peptides assembled at the bottom (light-exposed) window of the cuvette. (b) Detail of light-exposed cuvette window in channel containing 5.0 μM Pol-RFamide II (left), 0.6 μM He-RYamide (middle), and 1.2 μM He- RYamide (right): more than 90% of the larvae assembled at the window of the middle and right cuvettes.

All neuropeptides expected to exist in planulae of *H. echinata* were assayed for effects on photobehavior in planulae and young medusae of *T. cystophora*. According to the rationale outlined in Figures 1 and 2, the peptides listed in Table 1 were tested in concentrations ranging from 0.3 to 20 μM. The peptides were assayed by exposing larvae or medusae to unidirectional daylight in the

Table 1. Peptides studied in photo-behavior of planula larvae of *Hydractinia echinata* and of young medusae of *Tripedalia cystophora*

Peptide	Structure
He-LWamide I	pERPPGLW-NH_2
He-LWamide II	KPPGLW-NH_2
Pol-RFamideII	pEWLKGRF-NH_2
He-RYamide	EVLPLRRGRY-NH_2
He-RNamide	AESNGRHRNMIRN-NH_2

presence of externally added synthetic peptide. Each experiment had at least one control – medusae or planulae incubated in seawater without peptide supplement. Experiments were set up in late afternoon and evaluated at noon the next day in order to include day/night/day transitions.

Statistical analysis

For evaluation of phototaxis, number of specimens in the light-exposed middle third of the cuvette was compared with the number that assembled in the other two-thirds of it. For evaluation of locomotory behavior, number of specimens in the central third of the cuvette was compared with the number that had moved from the starting position. Either χ^2-analysis or Fisher's exact test was used to calculate significance.

Inhibitor treatment

Embryos of *H. echinata* were treated as in Plickert et al. (2003) by 20 μM diethyldithiocarbamate, an inhibitor of peptidylglycine α-hydroxylating monooxygenase (Mains et al., 1986). Treatment was started 20 h after fertilization and continued during the light-orientation experiment.

Results and discussion

RFamide and LWamide immunoreactivity in photoreceptive organs

Immunoreactivity was observed in close association with the ocelli or with the rhopalia that bear ocelli (Fig. 3). In none of the specimens could immunoreactivity be co-localized with regions where the photoreceptor cells of the ocelli occur or are supposed to be located (Fig. 3; Martin, 2002). In *Tripedalia cystophora*, RFamide immunoreactive nerve cells are located at the base of the rhopaloid bodies, close to the stalk insertion. They are distant from all six ocelli of the rhopalium but are closer to the two most lateral of the four simple ocelli than to either one of the complex eyes (Fig. 3c and d). As in the cubomedusa *Carybdea* (Martin 2002) and in hydromedusae (Grimmelikuijzen & Spencer, 1984), the ocellar nerve includes nerve cell processes that are RFamide-immunoreactive. At each of the four sites where the ocellar nerves join the ring nerve, a dense neuropilic area of immunoreactive processes and likely synapses was observed (NA in Fig. 3d). At these sites, photic information could be fed into the nervous system of the medusa. A comparable RFamide-immunoreactive neuropilic area is absent from the interradial insertion sites of the short tentacles of the young medusa. LWamide immunoreactivity was not observed in the optic nerve, but, comparable to the distribution of RFamide immunoreactivity, was observed at the base of the rhopaloid body (Fig. 3a and b). In addition, LWamide immunoreactivity was detected in the nerve plexus of the manubrium, the radial and ring nerves, and the neuropilic area at the base of the rhopalium stalk (not shown).

Freshly liberated ephyrae of *Aurelia aurita* showed intense RFamide immunoreactivity in nerve cells of the rhopalia. They were interconnected to the epithelial nerve plexus, which is also RFamide immunoreactive (Fig. 3e). LWamide immunoreactivity was, in contrast, restricted to nerve cells in the proximal half of the rhopalium. These cells are not coupled via LWamide immunopositive neural processes to the nervous system of the ephyra (Fig. 3f and g). Also in *Cladonema radiatum*, members of both peptide families are obviously expressed in the nervous system including the plexus of the manubrium and the radial and ring nerves. The ocelli of *C. radiatum* were not stained by antibodies to RFamide or LWamide. Ocellar nerves connecting the ocellus to the outer marginal nerve cord were contrasted by RFamide immunoreactivity but not by LWamide staining (not shown).

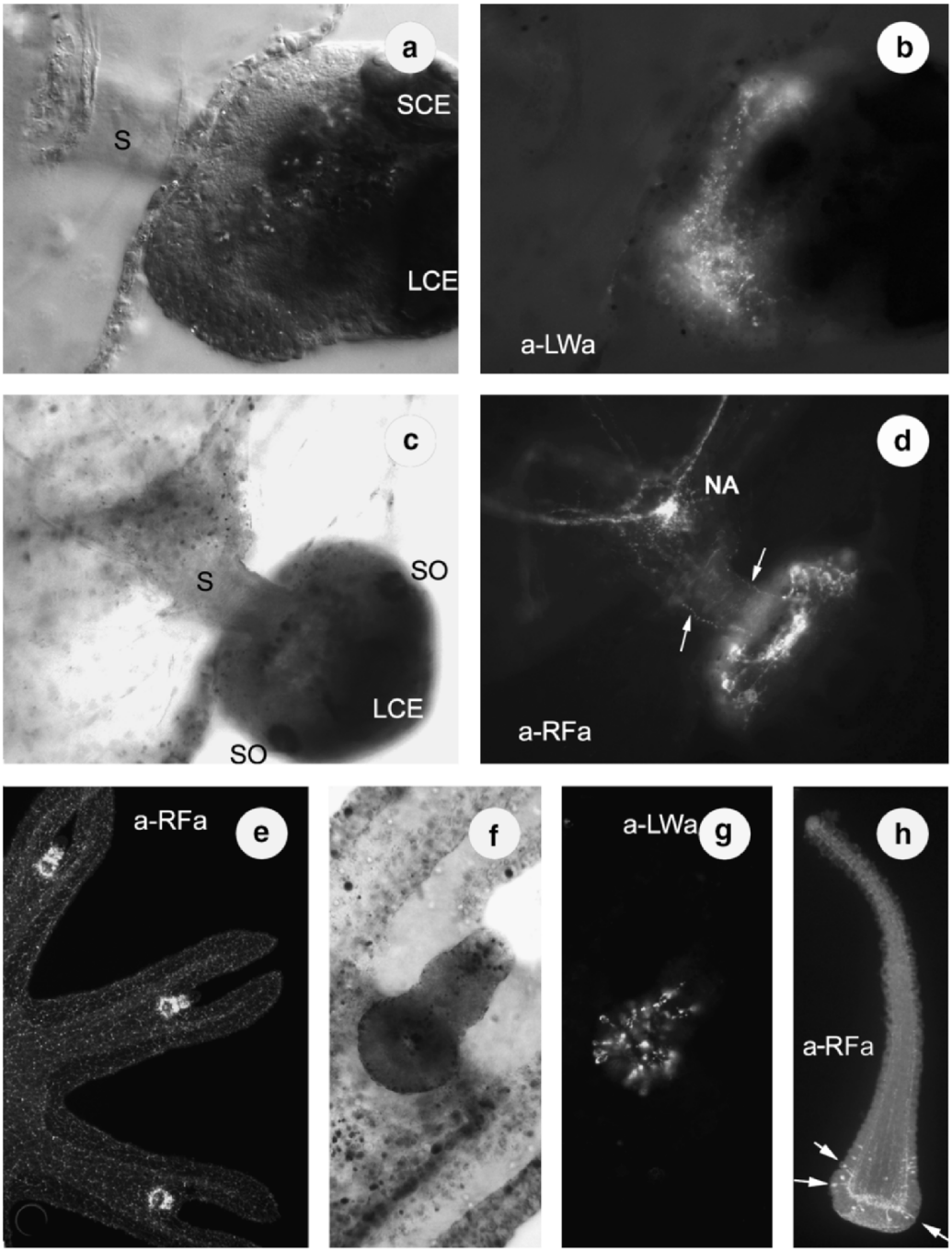

Figure 3. RFamide and LWamide immuoreactivity in photoreceptive organs of cnidarians and in the planula of *Hydractinia echinata*. (a, b) Bright-field and fluorescence views of LWamide immunoreactivity in rhopalia of *Tripedalia cystophora*. LWamide immunoreactive cells occur at the base of the rhopaloid bodies distant from both the large complex eye, LCE, and the small complex eye, SCE. (c, d) RFamide immunoreactivity occurs in a similar distribution. It is also absent from the ocelli but appears to be more closely associated with the lateral simple ocelli, SO, of the dimple type than is LWamide immunoreactivity. RFamide immunoreactivity is present in processes of the ocellar nerve (arrows in D) in the stalk, S, and in a neuropilic area, NA, where the ocellar nerve joins the ring nerve. (e) RFamide immunoreactivity in ephyrae of *Aurelia aurita*. Note strong reactivity in the rhopalia-bearing and photoreceptive organs. (f, g) Bright-field and fluorescence views of *Aurelia* rhopalium showing LWamide immunoreactivity in proximally located neurons of the rhopalium. Note missing interconnections by immunopositive processes to the nerve plexus of the ephyra. (h) RFamide immunoreactive neurons (some indicated by arrows) in anterior part of a planula. Images were acquired digitally by standard fluorescence, brightfield, and Nomarski/DIC microscopy.

Origin of peptides and assay for possible functions in photo-orientation

The peptides became known through two lines of evidence. Metamorphosin A (MMA), a peptide isolated from the sea anemone *Anthopleura elegantissima* induces metamorphosis in *Hydractinia echinata* (see Leitz & Lay, 1995). Molecular cloning revealed that *Hydractinia* has two endogeneous peptides similar to MMA, He-LWamide I and He-LWamide II (Table 1) (Gajewski et al., 1996). Since neurosensory cells of the planula (Fig. 3h) and other neural cells of *Hydractinia* are RFamide immunoreactive, the respective preproprotein was identified by cDNA-cloning (Gajewski et al., 1998). The *Hydractinia* RFamide, pEWLKGR- Famide, has the same structure as Pol-RFamide II from the hydromedusa *Polyorchis penicillatus* (Schmutzler et al., 1994). In *Hydractinia*, it is encoded in a multiple neuropeptide precursor together with at least two other peptides, He-RYamide and He-RNamide (Table 1) (Gajewski et al., 1998). RFamide immunoreactive nerve cells and LWamide immunoreactive nerve cells both occur in the anterior part of developing and mature larvae (Plickert, 1989; Leitz & Lay, 1995; Gajewski et al., 1996; Plickert et al., 2003; Fig. 3h).

Among the investigated neuropeptides, RFamide most strongly interferes with photic behavior and locomotion

Usually about 18 of 20 medusae assembled in the light-exposed third of the control cuvette while usually more than 130 of 150 planulae did so. Inhibition of phototaxis or locomotion is reported in Table 2 if distribution of the animals was significantly different ($p \leq 0.05$). RFamide inhibited photo-orientation in both investigated species (Table 2). While 0.6 μM Pol-RFamide prevented movement of *Hydractinia echinata* planulae toward a light source, inhibition of phototaxis in *Tripedalia* required a peptide concentration of at least 5 μM. At this and lower concentrations, locomotory action was not prevented in *Tripedalia*; the medusae swam actively by regular contractions of the bell that were not distinguishable from contractions in untreated control medusae.

Table 2. Effects of neuropeptides on young medusae of *Tripedalia cystophora* and on planula larvae of *Hydractinia echinata*

Peptide	Concentration (μM)	*Tripedalia*		*Hydractinia*		
		Phototaxis	Locomotion	Phototaxis	Locomotion	Metamorphosis
Pol-RFamide II	20.0, 10.0	–	–	–	–	–
	5.0	–	+[a]	–	+[a]	–
	2.5, 1.2, 0.6	+	+	–	+	–
	0.3	+	+	+	+	–
He-RNamide	20.0, 10.0	n.d.	n.d.	+	+	–
	5.0, 2.5, 1.2, 0.6	+	+	+	+	–
He-RYamide	20.0, 10.0	n.d.	n.d.	–	+[a]	–
	5.0, 2.5, 1.2, 0.6	+	+	+	+	–
He-LWamide I	20.0, 10.0	n.d.	n.d.	+	–[b]	+
	5.0, 2.5	+	+	+	–[b]	+
	1.2, 0.6	+	+	+	+	+
He-LWamide II	20.0, 10.0	n.d.	n.d.	–[b]	–[b]	+
	5.0, 2.5	+	+	–[b]	–[b]	+
	1.2, 0.6	+	+	+	+	+

[a] Reduced locomotory activity.
[b] Onset of metamorphosis causes halt in locomotion.
n.d.: Not determined.

At 5 μM RFamide peptide concentration, however, activity of swimming was reduced. While still locomotorily active, as proved by the fact that the animals had left the central area of the cuvettes, they were no longer able to steer toward the source of illumination. Immediate and complete arrest of rhythmic contractions of the bell was caused by 10 and 20 μM RFamide peptide. When the peptide solution was replaced with seawater, normal contraction behavior resumed. Locomotion of *Hydractinia* planulae was prevented at the highest concentrations (10 and 20 μM) of RFamide and was substantially reduced at 5 μM. Planulae dispersed from the start region of the cuvette but did not move to all possible areas as control animals did (indicated in Table 2 by an asterisk). At lower concentrations, larvae dispersed normally but more slowly. The RFamide also reduced activity in planulae by lowering the frequency of periods with locomotory activity (analysis by time-lapse recording, data not shown). In each species, He-RNamide did not have any obvious effect on photo-behavior or locomotion. He-RYamide, which is expected to be processed from the common precursor as the RFamide and the RNamide, prevented phototaxis and reduced planula locomotion in concentrations of more than 10 μM. He-LWamides are essential signals in metamorphosis (Gajewski et al., 1996; Plickert et al., 2003). Accordingly, they induced metamorphosis and thus stopped movement of the planulae in the cuvette (Table 2: 2.5, 5.0, 10.0, and 20.0 μM He-LWamide I and He-LWamide II). At lower concentrations, however, the planulae moved to the illuminated window of the cuvette before commencing metamorphosis. LWamides do not interfere with light-orientation.

Peptideamide knock-out eliminates phototaxis but not locomotion

To study the requirement of peptideamides (Table 1) for locomotion and phototaxis in planulae, we implemented a pharmacological peptide knock-out. C-terminal amidation is the rate-limiting step in peptide biosynthesis (Eipper et al., 1993) and is likely essential for receptor binding and activation (review by Kulathila et al., 1999). This final step of processing was targeted as described elsewhere (Plickert et al., 2003). Embryos of *H. echinata* were exposed to inhibitors of amidation from hour 20 of embryonic development and thus before expression of neuropeptide precursor genes of the investigated peptides had begun. As a consequence, larvae that developed from these embryos were depleted of any bioactive peptideamides, as Plickert et al. (2003) showed. Peptideamide-depleted larvae showed normal locomotory behavior. A minor difference was that velocity of locomotion was slightly reduced in comparison to controls. Neither of the amidated peptides present in normal larvae (Table 1) is thus required for a basal activity of the locomotory system. Generation of planula locomotory forces by contraction of epitheliomuscular cells or by beating of cilia is independent of any peptideamide. Phototaxis, however, was absent in treated planulae, indicating that at least one of the peptides knocked out is indispensable for the transmission of photic information to the locomotory system. Placed in the center of a light-orientation cuvette, larvae dispersed from that position randomly (like planulae in the RFamide treatment: Fig. 2a, 4 channels on the left).

A role for RFamides in photo-oriented locomotion of Cnidaria

The experimental results show that creeping locomotion of planulae does not require action of peptideamides as possible neurotransmitters. In contrast, reaction to light by steering toward a light source depends on amidated neuropeptides in the planulae. The results of this study are that among the peptides present in the larva, the *Hydractinia* RFamide Pol-RFamide II plays a major role. RFamide prevented photo-orientation in planulae 10× more efficiently than He-RYamide, while He RNamide in the investigated concentrations had no effect. Disturbance of photo-orientation by RFamide was also observed in young medusae of *Tripedalia*. That 10× more RFamide was required to disturb phototaxis in *Tripedalia* may be due to differences in peptide uptake. Alternatively, the endogenous RFamide of *Tripedalia* may be expected to have a different amino acid sequence than the Pol-RFamide II peptide used in this study. Therefore, Pol-RFamide II may be less active than the endogenous *Tripedalia* RFamide. Since higher concentrations paralyzed

Tripedalia medusae, RFamides must control photo-oriented locomotion by inhibitory action.

Hypothesis

We propose that RFamides play a key role in transmitting light stimuli to the epitheliomuscular system generating movements, contractions, and locomotion. We suggest RFamide immunoreactive cells function as interneurons between photoreceptor cells and swimming motor neurons in medusae or epitheliomuscular cells in planulae (and polyps). Stimulated by the photo-activated receptor cells, RFamide is released from synapses of the nerve processes of neighboring RFamide neurons. The released peptide inhibits activity of muscle entities downstream. Direct action on muscle cells in the simply organized larva is expected as well as indirect action involving neuromodulation of swimming motorneurons in medusae. In all nervous systems of medusae studied so far, RFamide immunoreactivity has been observed in radial nerves and thus in sectors of the bell. Also the other parts of the RFamide immunoreactive nervous system located around the circumference of the bell or around the manubrium could be part of functional sectors. Shading by the usually carotenoid-rich manubrium or pigmention of tentacle bulbs may create a difference in energy uptake between the light-exposed and the opposite side of the bell. Given that RFamide peptide release is proportional to the quantum absorption of photo-receptors that trigger RFamide neurons, peptide release will be greatest in light-exposed sectors. Due to the inhibitory action of RFamide, this will reduce locomotory action in that sector. As a consequence, the medusa tilts and turns toward the light source. This mechanism allows photo-orientation also in species without ocelli. The same principle is proposed to cause photo-orientation in planulae, in which the lateral ectodermal epithelia are expected to function as planar photo-receptive areas shielded from back-light by the carotenoid-rich endoderm. Unilateral illumination is therefore expected to cause increased release of RFamides from neurosecretory cells in the illuminated flank and consequently decrease of locomotory activity on this side of the animal. As actually observed, the larva, while creeping forward, bent the anterior part over the axis of light direction, exposing the previously unilluminated side toward the light source and causing bending in the opposite direction. By bending back and forth, the larva steered toward the light source.

The role of He-RNamide, He-RYamide and of He-LWamides

The *Hydractinia* precursor for Pol-RFamide II includes propeptide units for He-RNamide and He-RYamide (Gajewski et al., 1998). Therefore, the cells synthesizing RFamide are expected to process and release these peptides, too. The precursor includes 20 copies of Pol-RFamide II and only one copy each of He-RNamide and He-RYamide. Since no mechanism of differential release is known, concentrations of the peptides released from the neurosecretory cell will be proportional to their stoichiometry in the precursor. In this respect, the concentrations of He-RYamide and He-RNamide applied in the experiments must be considered to be physiologically 20× more active than a comparable treatment with the same molar concentration of the RFamide. Despite that, only concentrations greater than 5 μM of He-RYamide affected behavior of planulae (instead of 0.6 μM RFamide). From this we conclude that the endogenous He-RYamide has functions other than controlling phototaxis and locomotion. This function as well as the function of the RNamide is not known. LWamides occur in the same anterior part of the larva as RFamide. LWamides are mediator signals in metamorphosis (Leitz & Lay, 1995; Gajewski et al., 1996; Plickert et al., 2003). LWamide immunoreactivity was observed in association with photoreceptive organs and in the nervous system of Cnidaria. In the orientation assay, LWamides did not affect phototaxis. The function of LWamides at sites of immunoreactivity must be considered to be unknown.

Acknowledgments

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Different roles of GABA and glycine in the modulation of chemosensory responses in *Hydra vulgaris* (Cnidaria, Hydrozoa)

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Abstract

Phylogenetic studies suggest that GABA and glycine receptors derive, as a result of divergent evolution, from a common ancestral protoreceptor originated in a unicellular organism. This raises the possibility that members of the ligand-gated ion channels (LGIC) superfamily might be widely present in living organisms including bacteria and primitive invertebrates. High-affinity GABA receptors occur in the tissues of *Hydra vulgaris* whose pharmacological characteristics compare with those of mammalian ionotropic GABA receptors. Behavioural studies have shown that activation of these $GABA_A$-like receptors by their allosteric modulators increases the duration of response to reduced glutathione (GSH). Recently, strychnine-sensitive glycine receptors have been shown to occur in *Hydra* tissues. Activation of these glyR also results in increased duration of the response to GSH. In order to investigate the contribution of endogenous transmitters to the modulation of the feeding response, we studied the effects of exposing the polyps to brief depolarizing pulses prior to the GSH test. A severe inhibition of the response was observed following exposure to KCl or veratridine. Administration of GABA or muscimol counteracted the effects of the pulses in a dose-dependent manner. The effects of GABA or muscimol were suppressed by the $GABA_A$-specific antagonist gabazine both in pulse-untreated and treated polyps. By contrast, glycine and its agonist taurine were not able to restore the physiological duration of response in pulse-treated *Hydra*, while another glyR agonist, β-alanine, partially reduced the pulse-induced inhibition. We conclude that GABA appears to be the major inhibitory transmitter responsible for the regulation of the feeding response. Molecular studies aimed at identifying GABA receptor subunits are in progress.

Introduction

Aquatic animals rely largely on chemical senses for many biological activities ensuring survival such as food recognition and feeding, avoidance of predators, enhancement of reproduction, and recognition of suitable habitats. In the freshwater polyp *Hydra vulgaris* (Cnidaria, Hydrozoa) feeding is achieved through a complex behavioural pattern prompted by substances outflowing from wounded prey (Loomis, 1955). In the absence of prey some of these behaviours, e.g. tentacle writhing, tentacle concerting, and mouth opening, can be produced by polyps' exposure to reduced glutathione (GSH), which is the physiological stimulant of the feeding behaviour in *Hydra* as well as in other cnidarian species. Onset of response, i.e. mouth opening, occurs 1–2 min after GSH addition. Duration of the response, i.e. the time interval between mouth opening and mouth closure, is linearly related to the stimulus intensity in the low micromolar GSH concentration range. Different structural GSH analogs are able to inhibit GSH activity by preventing mouth opening. These

findings led to the hypothesis that a specific chemoreceptor mediated this response (see Lenhoff, 1974, for a review). Later studies by Venturini (1987) and Grosvenor et al. (1992) demonstrated the occurrence in *Hydra* tissues of GSH receptors that were further characterized, solubilized, and partially purified (Bellis et al., 1992, 1994).

The feeding behaviour is interrupted upon GSH removal, but the mechanisms by which termination of the response is achieved are still poorly understood. Lenhoff's suggestion (1974) that mouth closure simply derived from receptor desensitization, based on his detailed analysis of the kinetics of the response, was challenged by later studies. Grosvenor et al. (1996) found that wounded prey release other substances, besides GSH, that are able to shorten response duration by competitively inhibiting GSH binding. Our group previously reported that two inhibitory amino acid transmitters, GABA and glycine, modulate the feeding behaviour by prolonging duration of the response to GSH. Furthermore, glycine reduces duration of the response when all strychnine-sensitive binding sites are blocked by strychnine; this effect is mimicked by D-serine, a glycine agonist at the glycine-binding site of vertebrate glutamate NMDA receptors.

The action of these amino acids is mediated by receptors occurring in *Hydra* tissues whose biochemical and pharmacological characteristics compare with those of ionotropic mammalian GABA and glycine receptors (Concas et al., 1998; Pierobon et al., 2001). The inhibitory effect on response duration exerted by NMDA coagonists suggests that glutamate receptors also may be involved in the modulation of effector responses, besides the direct action of glutamate as a competitive GSH antagonist at the GSH receptor (Lenhoff, 1961). However, other studies (Bellis et al., 1991; Grosvenor et al., 1992) found that glutamate, which prevents GSH-induced mouth opening *in vivo*, did not displace the specific binding of radiolabeled GSH, and must therefore block the feeding response by a mechanism other than competitive inhibition. An increasing body of evidence thus supports the hypothesis that in *Hydra* duration of the response to GSH stimulation is finely tuned by GABA and glutamate with opposite actions, while glycine has a dual role. Since times of mouth opening after GSH administration are not affected, but only times of mouth closure vary because of treatment, the observed effects may well be the result of a coordinated interplay of different target cells (neurons?) activated by the GSH transduction pathway.

Materials and methods

Duration of the GSH response can be experimentally measured by recording times of mouth opening and closure for each animal, thus making the GSH test a reliable quantitative assay for studies of behavioural physiology. In order to investigate the contribution of endogenous GABA or glycine to the modulation of the response to GSH, we studied the effects of exposing the polyps to brief (15–20 s) depolarizing pulses prior to GSH administration. Animals were treated with 1 ml of 56 mM KCl or 1 μM veratridine (VTD), quickly washed with physiological solution buffered with 1 mM Tris–HCl (pH 7.4), and then assayed by the GSH test immediately, i.e. within 60 s after the pulse. The test was initiated by adding 1 ml of buffered physiological solution containing either GSH (1–10 μM) or GSH plus GABA, its agonists and antagonists, or glycine and its agonists at different concentrations. In all experiments three to five groups of animals were treated with GSH only and served as control. Details of methods and data analysis are described elsewhere (Pierobon et al., 2001). GSH, GABA, muscimol, pentobarbital, glycine, taurine, and β-alanine were obtained from Sigma (Milan, Italy). Gabazine, VTD, and tetrodotoxin (TTX) were obtained from Research Biochemicals (Milan, Italy).

Results and discussion

Effects of depolarization on the feeding response

A severe inhibition of the response was obtained in polyps exposed to 56 mM KCl for 15 s, quickly washed, and tested with 1–10 μM GSH. The duration of the response was significantly reduced starting at 4 μM GSH ($-18 \pm 1.2\%$); the maximal decrease was observed at 10 μM GSH ($-38.7 \pm 2.4\%$). Administration of the plant alkaloid VTD (1 μM), either by pulses or bath

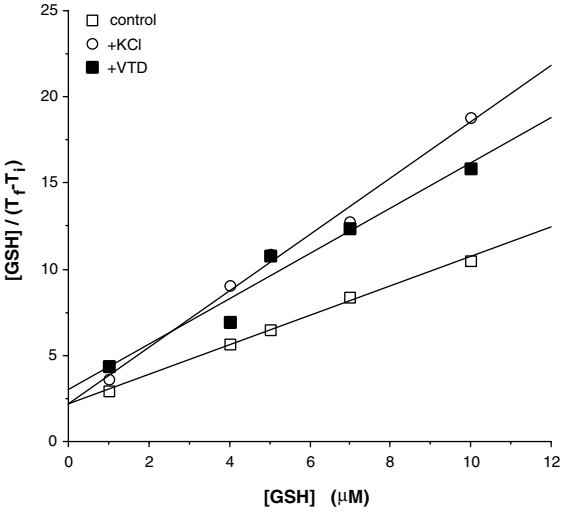

Figure 1. Linear regression curves of the response to different GSH concentrations (control) after 56 mM KCl or 1 μM veratridine pulses. Curves are obtained by a modified Lineweaver–Burk equation, where [GSH] represents the stimulus concentration and (T_f–T_i) is the time measured at the corresponding GSH dose.

applied, also produced a significant decrease in duration of the response (−36.7 ± 2.1% at 10 μM GSH) (Fig. 1). The reduced duration of response depended solely on anticipated times of mouth closure, while mouth opening was not affected by treatment (Table 1). In order to test the reversibility of the inhibition, in a different set of experiments the GSH test was performed at various times after the pulse. The effects of the pulse gradually attenuated starting 30 min after the KCl pulse. A complete recovery of response duration was observed 2 h after the pulse. Similar results were obtained in polyps exposed to VTD pulses (Fig. 2).

VTD or high KCl doses are commonly used in studies of transmitter release from neuronal preparations. Since in our experiments the entire animal underwent treatment, all excitable cells or cellular structures were simultaneously stimulated; therefore, the observed effects may well represent the composite result of a variety of cellular activities. Nonetheless, time to onset of mouth opening was not modified, suggesting that the binding ability of GSH receptors was not affected. Furthermore, duration of the response was still proportional to GSH concentration (Fig. 1). The observed decrease in response duration could then depend either on a reduced efficiency of the GSH signalling pathway or on a reduced ability of effector cells to respond to the stimulus (ion channel inactivation, onset of refractory periods, receptor desensitization, depletion of stored neurotransmitters). In the latter hypothesis, the GSH stimulus would reach its target cells after these have been activated by one or more transmitters released by the depolarizing pulse, thus being only partially effective. Alternatively, depletion of transmitter stores produced by the pulse may reduce availability of transmitters normally involved in the regulation of the effector response to the GSH stimulus. The time course of effect reversal is consistent with this model. The lack of an effect of pulses on the mechanisms of muscle contraction/

Table 1. Times of mouth opening and closing after GSH administration

Drug	T_i	T_f	T_f–T_i
Solvent	36″ ± 12″	21′58″ ± 1′13″	21′22″ ± 1′14″
56 mM KCl pulse	36″ ± 8″	13′10″ ± 1′31″	12′34″ ± 1′27″
1 μM veratridine	36″ ± 9″	13′35″ ± 1′14″	12′59″ ± 1′16″
100 μM GABA	33″ ± 13″	27′33″ ± 2′04″	27′00″ ± 2′08″
100 μM muscimol	40″ ± 10″	26′46″ ± 1′42″	26′06″ ± 1′41″
10 μM pentobarbital	36″ ± 8″	26′33″ ± 1′16″	25′57″ ± 1′16″
10 μM gabazine	44″ ± 14″	14′49″ ± 1′14″	14′05″ ± 1′21″

Times of response (minutes and seconds) after 10 μM GSH administration (T_0). Data are the means ± SD of individual times recorded in 8–10 separate experiments for each drug. T_i = time of mouth opening; T_f = time of mouth closure.
Duration of the response, i.e. the time interval (T_f–T_i), was calculated for each polyp in all sample groups. Average values were used for linear regression analysis or as a percentage of the control (ANOVA).

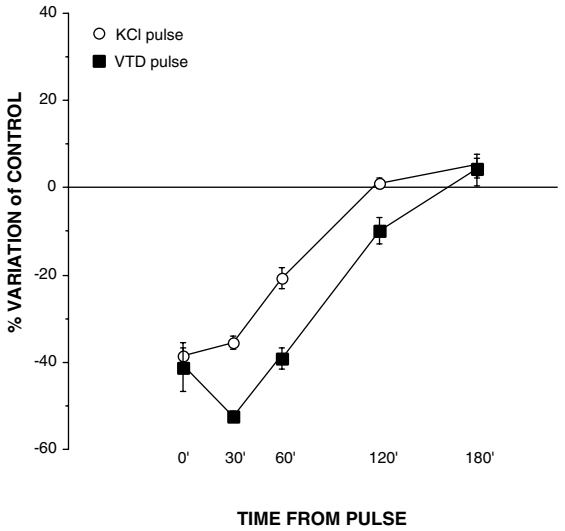

Figure 2. Recovery of times of response to 10 μM GSH at different times after KCl or VTD pulses. Data are expressed as a percentage of the control (10 μM GSH) value and are the means ± SEM from three to five separate experiments.

relaxation presiding over mouth opening (Campbell, 1987), though intriguing, is beyond the purpose of the present investigation and cannot be properly addressed here.

Effects of GABA, its agonists and antagonists

1 or 10 μM GABA, i.e. ineffective doses in KCl-untreated animals, counteracted the decrease in response duration in animals tested immediately after the KCl pulse. 100 μM GABA produced a significant enhancement of the GSH response that compared with the increase observed in KCl-untreated polyps (Table 2). The $GABA_AR$ agonist muscimol also prolonged response duration in a 10–100 μM concentration range. However, it was able to reverse KCl effects only at 100 μM but it failed to produce enhancement of the response (Table 2). Similar results were obtained in VTD-treated animals (Fig. 3). The increase in response duration induced by GABA and muscimol was suppressed by the simultaneous administration of the specific $GABA_AR$ antagonist, gabazine, in a dose-dependent manner (Table 3). Gabazine *per se* significantly reduced response duration in a 1–10 μM concentration range but did not produce a further inhibition of the response in KCl-treated polyps (Fig. 4).

The general anesthetic pentobarbital (100 nM–100 μM), a positive allosteric modulator of mammalian $GABA_A$ receptors, also produced a sig-

Table 2. Effects of GABA, glycine and agonists, alone or in combination with KCl pulses, on the duration of the response to GSH

Drug	Concentration (μM)	Response duration (% control)	
		Drug	56 mM KCl pulse + drug
Solvent		100.0 ± 4.4	61.3 ± 2.1*
GABA	1	105.3 ± 0.5	100.4 ± 4.5†
GABA	10	107.7 ± 3.2	103.3 ± 1.8†
GABA	100	127.4 ± 2.9*	123.3 ± 4.8†
Muscimol	1	100.2 ± 4.2	68.6 ± 3.0*
Muscimol	10	118.4 ± 3.2*	76.1 ± 3.7*
Muscimol	100	123.1 ± 3.1*	103.9 ± 2.6†
Pentobarbital	1	116.9 ± 3.1	75.5 ± 7.0*
Pentobarbital	10	122.4 ± 1.9*	75.9 ± 2.3*
Pentobarbital	100	120.9 ± 6.2*	57.0 ± 6.4*
Baclofen	100	107.6 ± 2.3	64.2 ± 1.5*
Glycine	100	124.9 ± 3.4*	63.5 ± 4.4*
Taurine	100	126.5 ± 3.0*	68.1 ± 1.8*
β-alanine	100	125.0 ± 2.3*	83.5 ± 1.5†

Data are expressed as a percentage of the control (10 μM GSH) value and are means ± SEM from five to seven separate experiments for each drug.
*$p < 0.001$ versus control; $^\dagger p < 0.001$ versus KCl-treated control group (ANOVA followed by Scheffé's test).

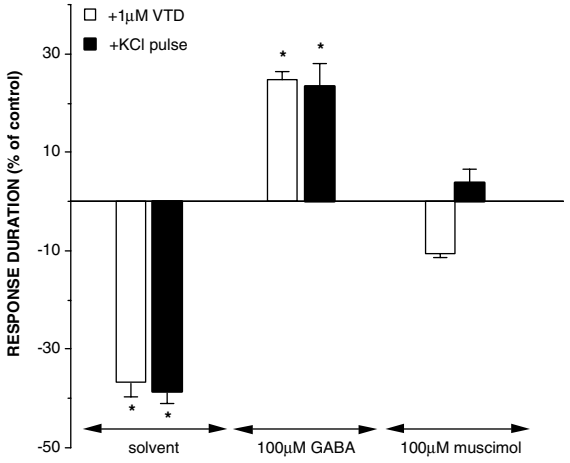

Figure 3. Effects of 1 μM VTD or 56 mM KCl on the duration of response to 10 μM GSH, in the presence or absence of 100 μM GABA or muscimol. Bars represent percent variations of control and are the means ± SEM from eight separate experiments (GABA) and from six separate experiments (muscimol). *$p < 0.001$ versus control.

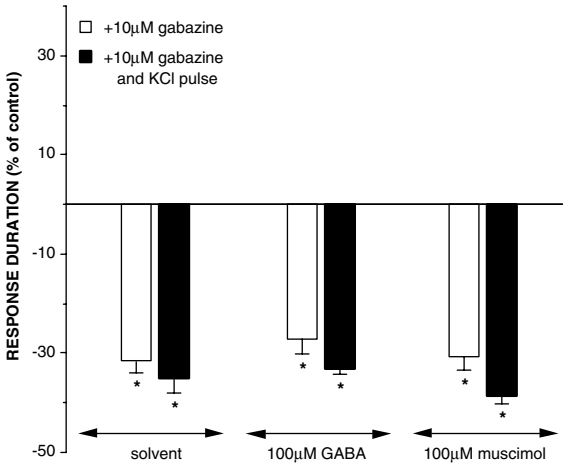

Figure 4. Effects of 10 μM gabazine on the duration of response to 10 μM GSH in KCl-untreated and KCl-treated animals, in the presence or absence of 100 μM GABA or muscimol. Bars represent percent variations of control and are the means ± SEM from eight separate experiments (GABA) and from six separate experiments (muscimol). Differences between KCl-untreated and treated groups are not significant. *$p < 0.001$ versus control.

nificant increase in response duration in KCl-untreated animals; the maximal increase was obtained by 10 μM pentobarbital (Table 2). Furthermore, co-administration of 100 nM pentobarbital and 1 μM GABA produced a significant increase in duration of the response to 10 μM GSH (+33.2 ± 5.1%). These effects were abolished by the simultaneous administration of gabazine (Table 3). Pentobarbital (1–100 μM) was completely ineffective in KCl-treated polyps (Table 2), while it counteracted the effects of bath applied VTD in a dose-dependent manner (Fig. 5). When polyps were treated with VTD pulses, only high (100 μM) pentobarbital doses were able to suppress the pulse-induced decrease of response (Fig. 5). The results obtained by GABA agonists and antagonists in this behavioural study are in good agreement with the results of previous binding experiments (Concas et al., 1998). Taken together these data also suggest a role for endogenous GABA in regulation of the feeding response. In fact, GABA, even at low concentrations, was able to restore physiological times of response in animals treated immediately after the depolarizing pulse. Muscimol mimicked the effects of GABA, though with lower efficacy. Since *Hydra* GABAR have a high affinity for the agonist muscimol (Pierobon et al., 1995), this finding could be explained with the hypothesis of a relative heterogeneity of GABAR populations. The effects

Table 3. Dose–response effects of gabazine, alone or in combination with GABA and agonists, on the duration of the response to GSH

Gabazine (μM)	Drug			
	Solvent	GABA (100 μM)	Muscimol (100 μM)	Pentobarbital (10 μM)
0.1	102.9 ± 4.4	134.3 ± 2.5*	128.6 ± 3.1*	127.7 ± 2.9*
1	95.0 ± 1.8	101.6 ± 3.8†	97.3 ± 2.4†	96.9 ± 2.7†
5	85.5 ± 2.2	88.7 ± 1.6†	88.9 ± 0.8†	66.7 ± 3.3†
10	68.4 ± 2.0*	72.9 ± 3.1†	69.3 ± 2.7†	60.2 ± 2.3†

Data are expressed as a percentage of the control (10 μM GSH) value and are means ± SEM from three to five separate experiments for each drug.
*$p < 0.001$ versus control; $^{\dagger}p < 0.001$ versus respective solvent-treated group (ANOVA followed by Scheffè).

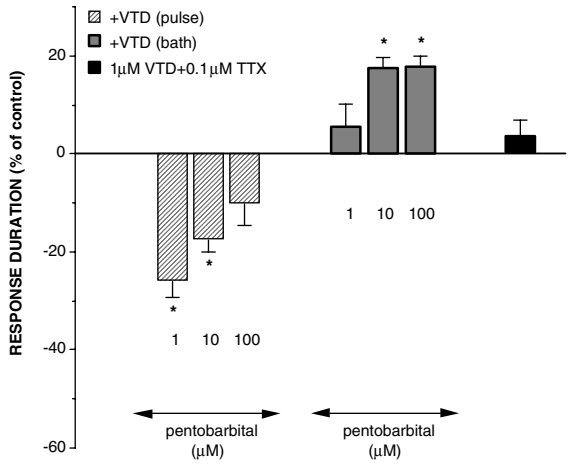

Figure 5. Effects of 1, 10, and 100 μM pentobarbital on duration of the response to 10 μM GSH after 1 μM VTD pulses or co-administration with 1 μM VTD. The effects of 1 μM VTD (see Fig. 3) are suppressed by 100 nM TTX; the difference is significant ($p < 0.001$). Bars represent percent variations of control and are the means ± SEM from four to six separate experiments. *$p < 0.001$ versus control.

of GABA or muscimol were suppressed by the specific $GABA_AR$ antagonist gabazine, indicating a receptor-mediated action. Pentobarbital, which potentiates [^{3}H]GABA binding *in vitro* (+78% at 300 μM pentobarbital), was only effective *in vivo* when animals were not subjected to KCl pulses. This result lends further support to the hypothesis that endogenous GABA may no longer be available as a consequence of the pulse, thus preventing pentobarbital potentiation.

By contrast, low pentobarbital levels were sufficient to restore or to increase response duration when co-administered with VTD, while high pentobarbital concentrations were required to attenuate the inhibitory effects of VTD pulses. In other organisms pentobarbital, besides potentiating GABA binding, is antagonistic to VTD, possibly by exerting a blocking action on voltage-gated, TTX-sensitive Na^+ channels, which are the primary target of a number of liposoluble neurotoxins (Wang & Wang, 2003). In our experiments the ability of pentobarbital to reverse VTD effects was not quite dependent on pentobarbital concentration, but rather on the timing of VTD administration. This finding is consistent with the hypothesis that the VTD-induced decrease of response could depend on the opening of a voltage-gated Na^+ channel. In fact, co-administration of 100 nM TTX and 1 μM VTD restored physiological times of response to 1–10 μM GSH (Fig. 5). Accordingly, pentobarbital would be able to antagonize the action of simultaneously applied VTD by competing onto a common target, while it would be substantially ineffective when VTD was pre-administered.

Finally the specific $GABA_BR$ agonist baclofen failed to modify the response either in untreated or in KCl-treated animals (Table 2). Instead, baclofen increases the probability of discharge of stimulated desmonemes (Kass-Simon & Scappaticci, 2004). In an electrophysiological study (Kass-Simon et al., 2003) bicuculline was found to suppress the decrease in frequencies of ectodermal and endodermal Contraction Pulses produced by GABA. In previous biochemical studies we have shown that [^{3}H]GABA binding is completely displaced by muscimol or gabazine, but not by bicuculline or baclofen, and it is potentiated by $GABA_AR$ positive allosteric modulators such as neurosteroids, general anesthetics and benzodiazepines. The evidence emerging from the bulk of these results supports the view that different GABA receptor populations are involved in the regulation of coordinated behaviours in *Hydra*, with different roles and specific pharmacological properties. Molecular studies aimed at identifying GABA receptor subunits are in progress.

Effects of glycine, taurine and β-alanine

Administration of 100 μM glycine or the glyR agonist taurine significantly increases duration of the response to GSH (Pierobon et al., 2001). However, when polyps were exposed to depolarizing pulses, both amino acids failed to restore physiological times of response (Table 2). In the presence of β-alanine, another glycine receptor agonist, a different result was obtained: duration of the response was significantly longer than that obtained after the KCl pulse, though still shorter than the control (−16.5 ± 1.5%). This partial recovery of response duration was suppressed by simultaneous administration of 10 μM gabazine (−34.7 ± 4.6%). The results obtained by β-alanine could be explained with the hypothesis of a modified molecular structure of *Hydra* glycine receptor subunits (Schmieden et al., 1993). Alternatively,

β-alanine is also known to act as a high affinity inhibitor of GAT-2 and GAT-3 GABA transporters (Cherubini & Conti, 2001). The suppression of glycine or taurine-induced enhancement of the response following depolarization could be tentatively explained with the hypothesis that modulation of the response to GSH by ionotropic glyR may require activation of GABAergic cells. It is interesting to note that co-administration with 1 μM gabazine suppressed the enhancement of response induced by either glycine or taurine in polyps not subjected to depolarization (Fig. 6). In biochemical experiments neither gabazine nor picrotoxin were found to displace [^{3}H]strychnine binding (-18 and -14% respectively in a 10^{-6}–10^{-4} M antagonist concentration range), thus excluding a direct action of $GABA_A$ antagonists on strychnine-sensitive *Hydra* glyR. By contrast, the action of GABA was not modified by simultaneous administration of 1 μM strychnine, the specific ionotropic glyR antagonist (Fig. 6).

Conclusions

Taken together, these results indicate that: (1) When the animal is exposed to KCl or veratridine pulses, it retains the ability to perceive the GSH stimulus and to react appropriately. However, the efficiency of the response is significantly reduced. (2) GABA and its agonist muscimol are able to restore or to enhance duration of the response in stressed animals, while pentobarbital is effective only in KCl-untreated polyps. (3) Suppression of the effects of GABA and its agonists by gabazine indicates that these effects are exerted through $GABA_A$ receptors. Furthermore, the effects of gabazine are not synergistic with those of KCl treatment. (4) Glycine and its agonist taurine do not affect the KCl-induced decrease of the response. Gabazine suppresses the effects of glycine and agonists in KCl-untreated polyps; this inhibition of glycine activity is not receptor-mediated, based on the results of [^{3}H]strychnine binding experiments. We conclude that GABA appears to be the major inhibitory transmitter responsible for the regulation of the feeding response, and that GABA and glycine act by different pathways.

The results obtained by the present approach, even though quite indirect, may begin to disclose the complexity of regulatory mechanisms underlying termination of the response to chemical stimuli in *Hydra*. Future studies on the cellular localization and regional distribution of these receptors will help to describe the circuitry involved and to understand the respective roles of inhibitory amino acid transmitters.

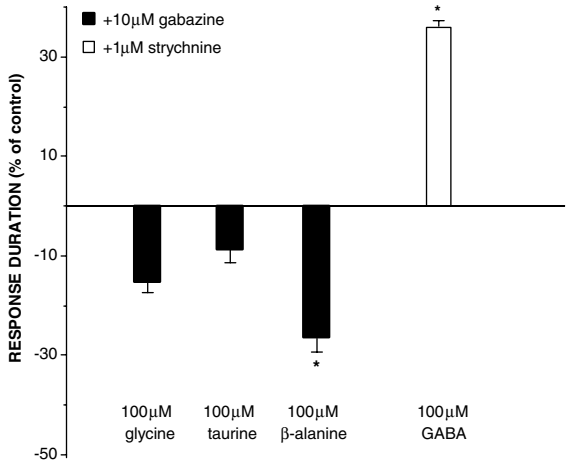

Figure 6. Inhibition by 1 μM gabazine of the enhancement of the response to 10 μM GSH produced by 100 μM glycine, taurine, or β-alanine. Co-administration of 100 μM GABA and 1 μM strychnine does not affect enhancement of the response. Bars represent percent variations of control and are the means $\pm$ SEM from four (glycine, taurine) to six (β-alanine) separate experiments. *$p < 0.001$ versus control.

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Glutamatergic and GABAnergic control in the tentacle effector systems of *Hydra vulgaris*

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Abstract

In addition to their role in orchestrating body and tentacle contractions, hydra's nerves control the behavior of nematocysts; precisely how is still a work in progress. There are strong indications that the classical neurotransmitters, glutamate and GABA (γ-amino-butyric acid), play an essential role in effecting stenotele and desmoneme discharge. In experiments on isolated tentacles of *Hydra vulgaris*, in which cnidocils were mechanically deflected with a piezo-electrically-driven glass micropipette, stenoteles and desmonemes respond to differences in applied force in a dose-dependent manner. GABA, working through its metabotropic receptor, appears to be involved with the recruitment of desmonemes. Desmonemes in distant battery cells or in another part of a given battery cell were discharged by stimulating a desmoneme cnidocil in the presence of bath-applied GABA or its metabotropic agonist, baclofen. The effect was blocked by phaclofen, its metabotropic antagonist. Neither GABA nor baclofen affected stenotele discharge. $GABA_A$ agonists had no effect on nematocyst discharge. Glutamate caused a significant increase in number of stenoteles responding to direct mechanical stimuli, but did not effect desmoneme discharge. The effect was mimicked by NMDA (*n*-methyl-D-aspartate) together with kainate, or by NMDA plus AMPA (amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid), but not with any ionotropic agonist alone. The effect was blocked by D-AP 5 (D- (−)–2-amino–5-phosphopentanoic acid), a specific NMDA antagonist, or CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), a specific kainate/AMPA antagonist. A glutamatergic mechanism working through ionotropic glutamate receptors appears to lower the firing threshold of stenoteles, perhaps by permitting the entry of Ca^{2+} into the cell through the early evolved NMDA/kainite/AMPA mechanism.

Introduction

Hydras have always been thought of as passive gentle feeders that sit quietly on *Elodea* blades waiting patiently for a daphnia or worm to blunder into their tentacles and brush against their nematocysts, which are then discharged. From the time of Parker (1919), Pantin (1942), and Ewer (1947), nematocysts have been tantalizingly thought of as independent effectors; that is, as cells that respond directly to a stimulus without the benefit of nervous intervention.

But the recent work of Watson & Hessinger (1989, 1991) and Thurm et al. (1998) has led to the suggestion that there are receptors in the tentacles of coelenterates that respond precisely to vibrations in the water in which they are immersed. Far from being passive, gentle animals, hydras, when they sense prey, become active predators. If one places a piezo-electrically-driven vibrating probe in close proximity to and within the whorl of outstretched tentacles of hydra head, as soon as the probe begins to vibrate, the tentacles approach the probe and attack it. The

vibrations transform the tentacles into predatory weapons.

The sensors that are likely to perceive the vibrations are those that reside in hydra's battery cell complex (Westfall et al., 1971; Hufnagel et al., 1985). The battery cell complex contains one or two sensory cells, a bipolar ganglion cell, and two sets of effectors: nematocysts and myonemes. Although the internal and external stimuli, which are integrated by the battery cell to control its effectors, are by no means yet defined, some headway has been made.

Response to applied force

The battery cell complex possesses many prey-ensnaring desmonemes but reportedly only one or two energetically-expensive toxin-bearing stenoteles. Stenoteles, therefore, must be conserved and cannot be spent frivolously – the probability of killing a prey has to be fairly high – whereas some of the many desmonemes can be expended more liberally in the interest of capturing a potential food object. One might expect, therefore, that as a consequence of these differences, desmonemes would be easier to discharge. We measured the responses of desmonemes and stenoteles to differences in applied force by stimulating individual cnidocils (which are clearly visible under the microscope) with single impacts of increasing force, administered with a piezo-electric-driven glass probe (see Cory & Hudspeth, 1980, for methods). The probability of eliciting either desmoneme or stenotele discharge is force-dependent; the response curve for both nematocytes is bell shaped; however, the minimum force that discharges stenoteles is 5× that needed to discharge desmonemes. Similarly, the optimal force, i.e. the force that has the probability of killing (or for desmonemes, capturing) the most prey, is 5× larger for stenoteles than for desmonemes.

Response to prey-substances

Although both stenoteles and desmonemes respond to single impacts of sufficient force, the chances of discharge are greatly increased if prey substances are included in the stimulus (Ewer, 1947); that is, if the object a hydra comes into contact with is something that smells or tastes like food. Mucin belongs to the group of N-acetylated sugars found on the external surfaces of several types of prey (Thurm & Lawonn, 1990; Watson & Hessinger, 1992).

If mucin is placed in the bath, it not only lowers the firing threshold of both desmonemes and stenoteles, but it effectively abolishes the dose–response curve for stenoteles, making weak impacts as effective as stronger ones, and lowering their response threshold to the minimum forces that discharge desmonemes without mucin treatment. This makes good sense because, presumably if a prey is nearby, any impact, weak or strong, is worth giving up a stenotele for, and so all stimuli can be permitted to be equally capable of making the stenotele fire.

Internal controls of nematocyst discharge

However, differences in force and chemical sensitization are not the sole determinants of nematocyst discharge. Battery cells have two types of nerves: sensory cells, whose apical ends project to the external medium, and ganglion cells, whose neurites traverse the length of the tentacle, at least to the next battery cell (Westfall et al., 1971; Yu et al., 1985: (Fig. 5e, f); Hufnagel & Kass-Simon, 1988), some of which possess an entire sensory apparatus, which would presumably be capable of perceiving tentacle vibrations and other perturbations to the tentacle. The nervous system, apart from its possible role as a modulator of nematocyst discharge (Kass-Simon, 1988; Westfall, 1988), is responsible for hydra's pacemaker output (Passano & McCullough, 1964, 1965; Rushforth & Burke, 1971; Kass-Simon, 1972, 1973; Kass-Simon & Passano, 1978).

In addition to the possible effects of peptides (e.g. Koizumi et al., 1989; Grimmelikhuijzen et al., 2002, for review) in modulating pacemaker activity in hydra as in other cnidarians (e.g. McFarlane et al., 1989), evidence has been mounting that the nervous system of hydra is a multi-transmitter system (Westfall, 1973; Westfall & Kinnamon, 1978; Kass-Simon & Passano, 1978) with some nerves perhaps producing more than one type of transmitter (Koizumi et al., 2004), as has been shown for the mammalian nervous system (e.g.

Walkade, 1998). Evidence for the involvement of classical neurotransmitters in hydra's coordinating systems is given in electrophysiological experiments (Kass-Simon & Passano, 1978; Kass-Simon et al., 2003). Glutamate was found to be generally excitatory, increasing the output of the body-contraction burst pacemaker and the rhythmic potential pacemaker (associated with the elongation of the body) as well as the tentacle-pulse pacemaker system. The effect is at least mediated by ionotropic kainate (tentacles and body) and AMPA (amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid) (body) receptors. GABA (γ-amino-butyric acid), working through its ionotropic receptors, on the other hand, is inhibitory. Biochemical studies support the electrophysiological findings. Binding studies have demonstrated reversible, saturable binding of both glutamate and GABA in membrane fractions of hydra (Bellis et al., 1991; Pierobon et al., 1995).

Glutamate and GABA also affect nematocyst discharge. Bath-applied glutamate increases the probability of stenotele discharge in a dose-dependent manner; it has no effect on desmonemes. Neither the ionotropic agonists kainate, AMPA, nor NMDA (*n*-methyl-D-aspartate) alone alters the probability of discharge. However, if either kainate or AMPA is administered with NMDA, the probability of discharge is significantly increased. (This coincides with the findings of Pierobon et al., 2001, of a non-strycnine-sensitive glycine binding site in hydra's membranes.) The effect on stenoteles is dose-dependent and blocked by either CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) or D-AP 5 (D- (−)–2-amino–5-phosphopentanoic acid), the respective antagonists of AMPA/kainate and NMDA. This fits in well with the idea that discharge is a calcium-dependent exocytosis (Skaer, 1973; Gitter & Thurm, 1993; Gitter et al., 1994), since the NMDA/kainate-AMPA mechanism would lead to an influx of calcium into the nematocyte, at the same time also removing calcium from the intracellular spaces between nematocyst and nematocyte.

Immunocytochemistry

Immunocytochemical findings support the physiological observations. Polyclonal antibodies to glutamate stain the cell bodies and axons of both sensory and ganglion cells (Hufnagel et al., 1993; Hufnagel & Kass-Simon, 1995), and antibodies to the ionotropic receptor sites label the nematocytes in a battery cell complex (anti GluR2/R3) and myonemes (anti-GluR1; Hufnagel et al., 1997, 1999, unpublished findings). NMDA receptors have been localized around desmonemes, stenoteles, and the myonemes of battery cells, as well as in nerves and several other cell types (Scappaticci et al., 2004).

With respect to the effects of the inhibitory amino acid, GABA, on nematocyst discharge, GABA, working through its $GABA_B$ receptors, promotes the distant discharge of desmonemes. That is, when GABA or the $GABA_B$ agonist baclofen is added to the bath, desmonemes other than the one that has been directly stimulated can be made to discharge. The indirectly stimulated desmonemes can be either in the same battery cell or in adjacent battery cells. The effect is abolished by the $GABA_B$ antagonist phachlofen. $GABA_A$ agonists have no effect on desmoneme discharge; nor are stenoteles affected by any GABAnergic ligands. GABA's ionotropic effects on the pacemaker systems are inhibitory, causing a decrease in pacemaker output (Kass-Simon et al., 2003).

Fluorescent labeling with anti-$GABA_B$ receptor antibodies reveals $GABA_B$ receptors in nematocytes of desmonemes and stenoteles, while localized anti-GAD antibodies reveal the presence of glutamatic acid decarboxylase (GAD) in epitheliomuscular cells. What this implies is that (a) desmoneme-containing nematocytes appear to be behaving like sensory cells, as Thurm and coworkers have suggested for stenotele-containing nematocytes (Brinkman et al., 1995; Thurm et al., 2004), and (b) $GABA_B$ receptors are involved in inhibiting previously inhibited desmonemes, so that GABA effectively lowers their firing threshold to zero, and thus permits desmonemes to be recruited in an attack when one of their number has encountered a good-tasting – for example, a mucin-exuding – prey object.

Summary and conclusion

The scenario that is beginning to evolve is this: vibrations in the medium (probably sensed by the

sensory cell or perhaps also by the ganglion cell) cause the tentacles to approach the interesting moving object. If the object also tastes good, i.e. exudes N-acetylated sugars (mucin), a desmoneme will be discharged; this will cause other desmonemes to be recruited by some mechanism involving the GABAnergic disinhibition of previously inhibited desmonemes. At the same time, mucin lowers the firing threshold of stenoteles, whose threshold of which may already have been lowered by a synapsing glutamate-containing sensory cell (Westfall, 1973; Hufnagel & Kass-Simon, 1988; Hufnagel et al., 1999) which was excited in the first place by the vibrations in the water. Thereupon the stenotele discharges and pierces the prey …; and the rest is reduced glutathione history (Loomis, 1955; Lenhoff, 1981, for review).

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Epithelial conduction: recent findings, old questions, and where do we go from here?

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Key words: hydromedusa, jellyfish, excitable epithelia

Abstract

Work on the roles of excitable epithelia in hydromedusan behaviour is reviewed from the historical point of view. Of special interest are cases where epithelial impulses excite or inhibit nerves. It is suggested that nerves running in the endoderm canals as described in several species with excitable endodermal epithelia may function to convey excitation to nerves in the ectoderm, but the precise pathways and mechanisms whereby epithelial depolarizations lead to the generation or suppression of neurally mediated processes are still poorly understood. Recent work on feeding in *Aglantha digitale* shows a component (lip-flaring) that may be a version of the crumpling behaviour seen in other medusae. The pathways mediating feeding and crumpling behaviours are reviewed.

Introduction

In this paper, I will describe some of the early findings and ideas about epithelial conduction, how they have come to be modified in the light of subsequent work, and what still remain as outstanding questions facing future workers. I say 'future' because, after an initial flurry of activity when the phenomenon came to light in the 1960s, lasting into the 1970s, most cnidarian neurophysiologists (including the writer) switched to working on other things. It is impossible however to get a proper picture of behaviour in most hydrozoans without taking excitable epithelia into account, and much remains to be investigated in this field.

We are talking here about electrical impulses that propagate on an all-or-none basis in epithelia, going from cell to cell via gap junctions, and bringing about effector responses at points that are often distant from the site of impulse initiation. The action potentials are basically sodium spikes, but often have a calcium component and in some cases can use calcium alone for the inward current in experimental conditions. All of these epithelia can act as sensors, responding to touch, and some of them double as effectors. For example, the striated swimming muscle in many hydromedusae and siphonophores is an excitable myoepithelium that conducts the impulses for its own contraction. (Myoid conduction is of course widespread in vertebrates too, in cardiac and many smooth muscle systems, but these are three dimensional structures rather than epithelia.). Other conducting epithelia lack any known effector capability but serve merely as signaling pathways, acting rather like nerves in this respect. Where, however, nerves typically innervate specific effectors in a highly directional and selective manner, conducting epithelia spread their impulses widely to effectors on all sides.

Conducting epithelia are two-dimensional sheets of cells, one cell thick. From the point of view of their cable properties, they are intermediate between nerves, which are one-dimensional, and cardiac muscle, which is three dimensional. Other things being equal, they will be harder to

excite and conduct more slowly than neurons of similar cross-sectional dimensions, as current will flow out radially in all directions within the cytoplasm and current density will fall off more rapidly (see Josephson, 1985). The action potentials often 'fatigue' rapidly, declining in amplitude and conduction velocity. In one case, this has been linked to progressive build-up of calcium ions in the cytoplasm (Grigoriev & Spencer, 1995). Another difference is that hydrozoan excitable epithelia are not known to form chemical synapses. They clearly affect the activity of nerves in their vicinity, either inhibiting or exciting them, but how they do so is not understood, and is one of the things touched on here.

The older work on excitable epithelia in hydrozoans has been reviewed several times, most recently by Anderson (1980) and Josephson (1985), and is covered in book chapters dealing with hydrozoan behaviour such as Spencer & Schwab (1982) and Satterlie & Spencer (1987). No attempt will be made to present a complete picture here. The phenomenon of epithelial conduction is not confined to hydrozoans, as non-nervous conduction occurs in several other phyla and plays a major role in the behaviour of pelagic tunicates such as salps. Recently, hexactinellid sponges were added to the list (Leys & Mackie, 1997; Leys et al., 1999), although the conducting tissues here are not strictly speaking epithelia, but three dimensional networks. It is interesting that anthozoans and scyphozoans (including Cubozoa) can apparently get along without conducting epithelia of any sort.

Purely epithelial pathways

Some effector reponses in hydrozoan medusae and siphonophores appear to be conducted by epithelia alone. These include some of the first examples that came to light in the 1960's, such as the spread of blanching (opacification of the mesogloea) in swimming bells of *Hippopodius hippopus* Forskål (Mackie, 1965; Mackie & Mackie, 1967; Bassot et al., 1978). This response, which involves the sudden appearance of opaque granules in the mesogloea underlying the excitable exumbrellar epithelium, is probably due to a configurational change in a mesogloeal protein brought about by ionic changes resulting from passage of an impulse in the epithelium. The response is spread by all-or-none propagated impulses and, as a result, the whole animal goes from transparent to white. This is part of a suite of defensive reactions. Seen in the dark, the surface of the animal emits light, once again as a result of impulse propagation in the exumbrellar epithelium. Here however the photochemical response is intracellular. Accompanying blanching and light emission and like these responses triggered by propagated epithelial impulses, the cells of a glandular structure, the rete mirabile, swell up and release secretory droplets (Mackie, 1976).

In all these cases, we knew that the epithelia were excitable and we doubted that nerves were involved chiefly because nerves appeared to be completely absent from the epithelia in the excitable pathway. Secondly, elevating the concentrations of Mg^{2+} and other divalent cations that compete with Ca^{2+} to levels that blocked neurally-mediated responses had little effect on epithelial conduction itself, so Mg^{2+} sensitivity came to be seen as diagnostic of neural mediation. However, this was a red herring, and we now know that nervous conduction itself is often relatively immune to elevated Mg^{2+} levels, and impulses can spread through nerve nets where the units are interconnected by gap junctions, as it does in epithelia. At the effector end, responses are frequently diminished or blocked by Mg^{2+} regardless of whether the excitation pathway is nervous or non-nervous. However, both arguments applied in the cases mentioned above and seemed at first to apply to the 'crumpling' response of jellyfish such as *Sarsia tubulosa* M. Sars (Mackie & Passano, 1968) and *Stomotoca atra* A. Agassiz (Mackie, 1975). Here, excitation propagates in the exumbrellar epithelium and on reaching the margin jumps across to the subumbrellar endoderm layer (another non-nervous, excitable epithelium), spreading over it in the same way as in the exumbrella. One more 'jump' was required to fire the effectors, which were the radial muscles in the subumbrellar ectoderm. Impulses would then propagate epithelially through the radial muscles.

In much of our early work, it seemed unnecessary to suppose that nerves were involved in crumpling because electron microscopy showed transmesogloeal interconnections between the ectoderm and the endoderm in all the appropriate

places. We assumed these were the conducting bridges called for by the physiology. In another jellyfish, *Euphysa japonica* Maas, similar arguments were advanced to account for the spread of bioluminescence that accompanies crumpling. Excitation was found to jump from the ectoderm to the endoderm, presumably by transmesogloeal bridges, and to propagate in the latter, evoking light production. Excess Mg^{2+} blocked light emission but not the propagation of epithelial impulses themselves (Mackie, 1991). At least in some cases it still appears reasonable to suppose that transmesogloeal epithelial bridges serve as conduction links between excitable epithelia, although their mere presence is no guarantee of it, and direct proof of their involvement is still lacking.

The early ideas can therefore be summarized diagrammatically as a set of purely non-nervous epithelial pathways firing effectors located within, or closely associated with, the excitable epithelia themselves (Fig. 1). Secretion by the rete mirabile and a number of other epithelially mediated responses have been omitted for simplicity. With the exception of the excitation of the crumpling muscles, discussed further below, this summary still represents a viable model.

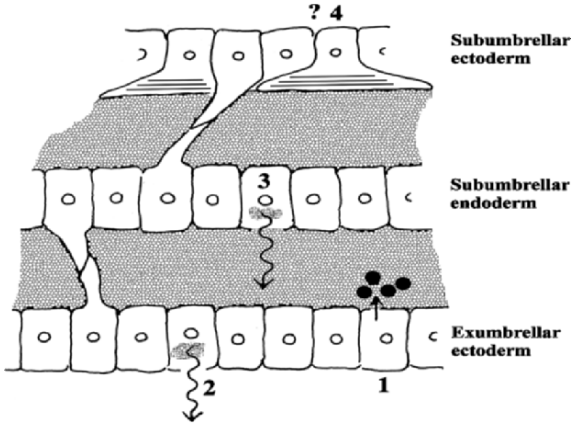

Figure 1. Composite diagram summarizing some early concepts regarding pathways and effector responses mediated solely by excitable epithelia (see text). All three epithelia were assumed to be capable of impulse propagation via low resistance pathways between the cells. Transmesogleal epithelial processes conducted impulses between layers. Effector responses: 1, production of opaque granules in the mesogloea (*Hippopodius*); 2, Light emission from exumbrellar epithelium (*Hippopodius*); 3, Light emission from endoderm lamella (*Euphysa*); 4, Activation of radial (crumpling) muscles (*Stomotoca*). The **?** indicates that this part of the story, always dubious, was effectively demolished by King & Spencer (1981). See Fig. 4 for an alternative explanation.

Pathways involving both epithelia and nerves

Some findings from the early work suggested that nerves were also involved in responses spread by excitable epithelia. In *Hippopodius* for instance, which has its own version of the crumpling response, the animal also stopped swimming when its exumbrella was excited. As swimming was known to be generated by pacemaker nerves in the margin, it seemed likely that the inhibition was due to some effect of epithelial excitation on the ability of the swim pacemaker neurons to produce action potentials. This was not obvious in the case of *Nanomia bijuga* Della Chiaje however. Here exumbrellar impulses did not block swimming but merely excited radial muscles in the velum whose contraction deformed the velum and altered the direction in which the water jet was emitted during swimming, causing the animal to swim backwards instead of forwards (Mackie, 1964). Some of the contradictory results from different siphonophores have now been resolved and it is quite clear that there are two-way epithelio-neural transition steps in many cases (Mackie & Carré 1983; Mackie et al., 1987) and that in many cases excitable epithelia collaborate with nerves in producing the behaviour.

Further study of solitary hydromedusae showed that here, as in the colonial *Hippopodius*, epithelial impulses could inhibit swimming. In *Stomotoca atra,* Mackie (1975) showed that the inhibitory action was on the neuronal pacemakers themselves rather than at some point in the motor pathway to the muscles and, in *Polyorchis penicillatus* Eschscholtz, Spencer (1981) showed that the neurons undergo long-lasting hyperpolarizations on the arrival of epithelial impulses. In *Aglantha digitale* Müller, a residual inhibitory effect was apparent even after swimming had been resumed, as shown by depression of the swimming frequency (Mackie et al., 2003). In contrast to these inhibitory effects, other responses appeared to reflect an excitatory effect on nerves, for instance in *Sarsia tubulosa*, where tentacular contractions, which are clearly neurally evoked, were found to accompany crumpling (Mackie & Passano, 1968).

The assumption that the contractions of the radial muscles during crumpling were mediated by cell-to-cell conduction in the muscles themselves had always been suspect because the contractions were known to be blocked in 100 mM Mg^{2+}, which does not affect epithelial conduction, suggesting a process of neural excitation through chemical synapses. The idea that nerves were not involved came under devastating fire from work on *Polyorchis* by King & Spencer (1981) who showed that epithelial impulses must somehow 'get into' nerves associated with the crumpling muscles before the latter can show contractions, and they were able to pin-point the locations where these transition steps were located.

Bearing in mind the *Polyorchis* work, I believe it would be useful to revisit *Stomotoca*. In both animals, we have the situation where the radial muscles are able to contract all together around the whole bell in one response (crumpling) and selectively in specific quadrants in another, the 'pointing' of the manubrium toward sites of captured food (Fig. 2). As there is no evidence of epithelial involvement in pointing, it seems clear that excitation is entirely neural in this case. We can suppose then that during crumpling the same nerves serve to relay excitation from adjacent excitable epithelia. The role of the conducting epithelium, then, would not be to excite the muscles directly as formerly supposed, but to excite the nerves that innervate these muscles (Fig. 3A). One way or another, the crumpling response owes its generality to the ability of the epithelial impulses to spread rapidly to all quadrants.

Aglantha can give us some pointers in this respect. It has what we consider to be an unusual variant of the crumpling response (Mackie et al., 2003). This jellyfish cannot crumple in the usual sense and lacks the radial muscles that produce the involution of the margin which is the basis of normal crumpling. It can however point, and retains radial muscle bands in the wall of the manubrium and lower part of the peduncle which bring this about (Fig. 3B). The neural pathway that we think serves for both crumpling and pointing is probably common to all medusae which show these responses, and is identifiable in several cases as a system of FMRFamide-immunoreactive neurons that run from margin to manubrium along the radial strands.

The muscles then can be excited in two ways in *Aglantha*. In pointing they are excited selectively by nerves originating in sensory cells in the tentacles and at the margin, leading to unilateral flexions of the manubrium. During ingestion of food however they are excited simultaneously in all eight sectors by epithelial impulses propagated in the endodermal radial canals, and this results in a symmetrical flaring of the manubrial lips. Lip flaring in *Aglantha* is evidently the counterpart of crumpling in other medusae. In the early stages of feeding when *Aglantha* has caught some food and has brought it in to the margin, epithelial impulses travel centripetally up the canals causing the lips to flare in anticipation of contact with the food. Later, during ingestion, flaring is again seen, this time generated locally in the manubrium itself. The flaring helps the lips advance around the food

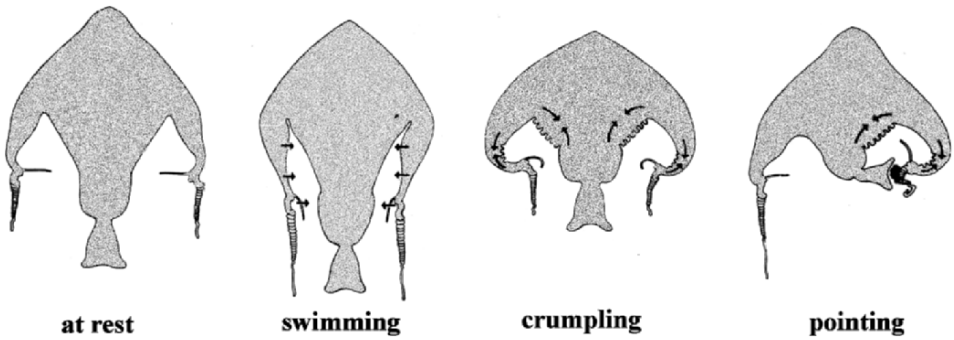

Figure 2. Activities of *Stomotoca* (after Mackie & Singla, 1975). Swimming is due to contractions of the circularly-orientated striated muscle fibres lying in the ectoderm of the subumbrellar cavity. Crumpling is brought about by contraction of radially-oriented, smooth muscle fibres that also lie in the subumbrellar ectoderm. Crumpling is a symmetrical response in which the radial muscles all the way around the bell contract simultaneously. Pointing, seen during feeding behaviour, represents unilateral contractions of the same radial muscles that bring about crumpling.

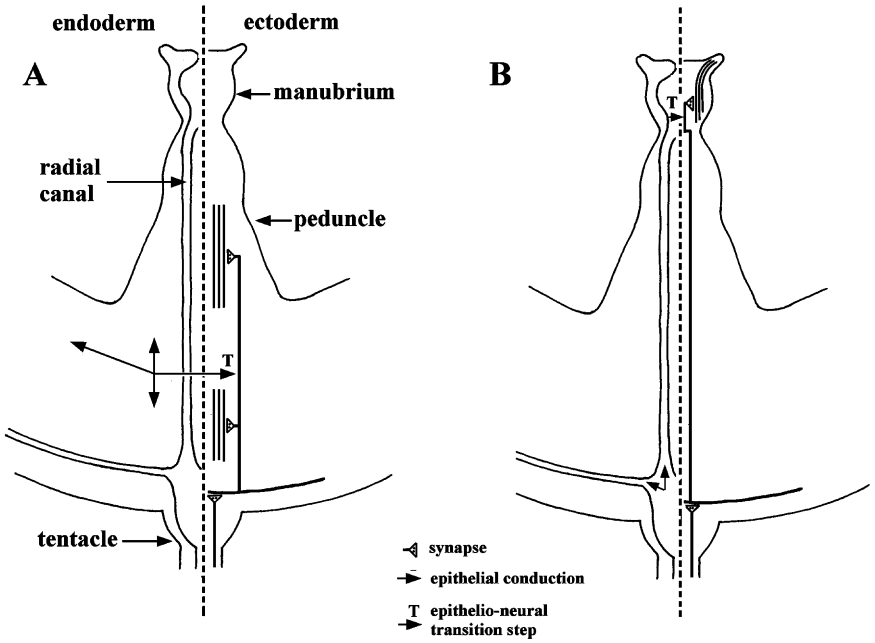

Figure 3. Present concepts regarding the organization of radial responses in a conventional medusa such as *Stomotoca* compared with the arrangement in *Aglantha*. In *Stomotoca* (A), pointing is due to selective excitation of individual radial muscle blocks (shown as groups of parallel lines) by nerves originating in the margin and tentacles. Crumpling is spread around the bell in all directions by epithelial impulses (arrows) and affects all groups of radial muscles. Epithelial impulses propagate throughout the endodermal lamella and radial canals. The final step in the activation of the muscles is shown as mediated by nerves, after an epithelio-neural transition step (T). *Aglantha* (B) cannot crumple but has radial muscles in the manubrium that are under dual excitatory control like those of *Stomotoca*. It can point unilaterally when local groups of radial muscles are excited via a nervous pathway. Epithelial impulses propagate in the endodermal canals. On reaching the manubrium, they bring about a symmetrical lip-flaring response, after an epithelio-neural transition step (T). Lip flaring is seen as a behavioural homologue of crumpling. In both animals, epithelial impulses are also propagated in the exumbrellar ectoderm. This is not shown, nor is the swimming circuitry, nor the inhibition of the swimming pacemakers by epithelial impulses.

object. Now, impulses are propagated centrifugally down the canals to the margin and inhibit swimming. *Aglantha* has evidently retained the essential excitable pathways, both nervous and epithelial, that it inherited from a common ancestor with the more conventional, crumpling medusae, and has redeployed the epithelial pathway to assist the processes of food ingestion. The inhibition of swimming which accompanies ingestion no longer serves a defensive role, but simply makes it easier for the animal to transfer and ingest food.

How do epithelial depolarizations 'get into' the nervous system?

It will not have escaped the notice of an alert reader that the lip-flaring, swim-inhibiting process in *Aglantha* must involve transmesogloeal crossings ('jumps') at both ends. Impulses arriving in the manubrium via the (endodermal) radial canals must somehow cross to the ectoderm where the flaring muscles are located. If the process works like the crumpling response of *Polyorchis*, we must assume that excitation of the muscles requires nervous mediation. Likewise, generation of flaring impulses during ingestion probably reflects the action of neural pacemakers, as it is part of a whole complex of locally generated, rhythmic movements, but somehow this activity 'gets into' the endoderm. Once in the endoderm, the impulses propagate centrifugally on a non-nervous basis, and when they reach the margin they must cross into the ectoderm in order to inhibit the swimming pacemaker neurons.

We must therefore ask where and how these two-way epithelio-neural transition steps take

place. As to where they take place, it could be either in the ectoderm or in the endoderm. Nerves have been seen in the endoderm canals by electron microscopy in *Sarsia*, *Euphysa*, *Stomotoca,* and *Polyorchis* (Jha & Mackie, 1967; Mackie & Singla, 1975; Spencer, 1979), all of which show the crumpling response. Nerves are reported to be absent from the endoderm only in *Nanomia* (Jha & Mackie, 1967), which has no crumpling or pointing response. The reason for their existence in crumpling species might be to 'pick up' epithelial depolarizations and convey them across the mesogloea to the ectoderm (Fig. 4). Unfortunately, although an endodermal nerve net can be well visualized by fluorescence microscopy (Fig. 5), no-one seems to have shown nerves actually crossing the mesogloea in these regions but such crossings might be few and far between and have escaped notice. It would be worth looking harder for them.

Nerves occur in the endoderm of the manubrium in *Aglantha* and could pick up the incoming epithelial depolarizations in the lip-flaring pathway but, again, they have not been seen crossing the mesogloea. Alternatively, epithelial processes might connect the ectoderm with the endoderm at the appropriate sites, and the two-way neuro-epithelial transition steps would then occur in the ectoderm, where nerves are much more numerous. Transmesogloeal epithelial bridges can usually be found in the appropriate places.

As to how the excitation process takes place, we may assume that nerves excite conducting epithelia through chemical synapses. This is certainly so in the case of the excitation of the swimming muscles (an excitable myoepithelium) and is probably true for simple, non-muscular epithelia like those of the endodermal canals. In salps, neuro-epithelial synapses and synaptic activity has been demonstrated, where the epithelium is a simple signaling pathway without an effector component (see in Anderson, 1980). We should look then for synapses between nerves and excitable epithelia in places where we know transition steps to occur.

As to how epithelial depolarizations excite or inhibit impulses in nerves, there is a virtually complete lack of solid evidence. Nothing equivalent to the epithelio-neural junctions (functionally synapses) found at attachment plaques between blastozooids in salp chains have yet been found in hydrozoans (see in Anderson, 1980). In *Polyorchis*, the epithelial impulses hyperpolarize the swimming pacemaker neurons, inhibiting swimming for up to several seconds (Spencer, 1981). Such a long-lasting event might suggest involvement of a slow transmitter, possibly a peptide. Peptides of epithelial origin have been shown to affect neural differentiation in hydra (Koizumi, 2002) and might function in medusae as neurotransmitters. The apparent lack of localized epithelio-neural synapses might reflect a process of diffuse transmitter release. Other possibilities include some sort of Mauthner cell-like external electrical field effect or alteration of the ionic environment surrounding the nerves (Mackie, 1975; Spencer, 1979). The thinking here is that neurons in the nerve rings of medusae are often roughly enveloped by processes

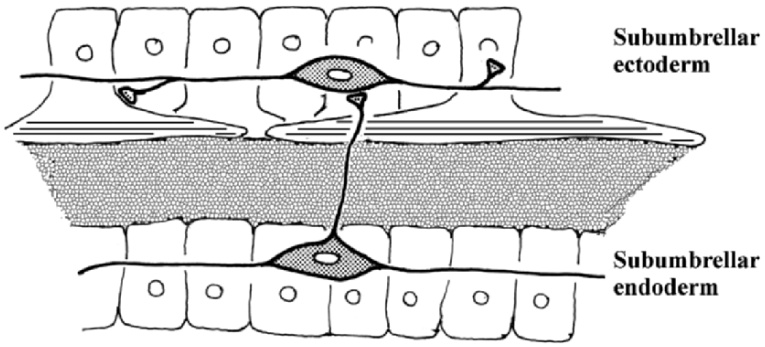

Figure 4. A possible role for the endodermal nerve plexus in forms like *Stomotoca*. Impulses propagated in the subumbrellar endoderm transduce impulses in endodermal neurons that cross the mesogloea, connecting with a nerve plexus in the ectoderm that excites the radial muscles in the crumpling response.

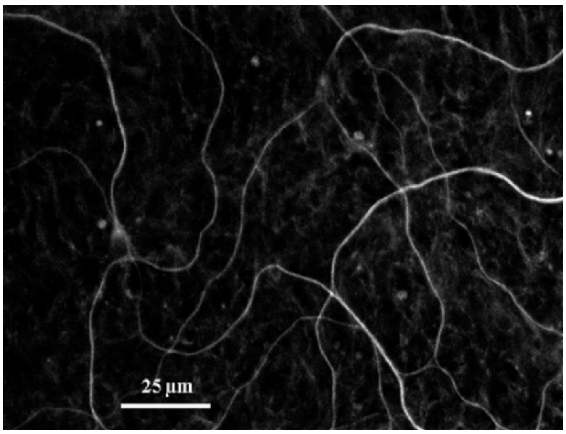

Figure 5. Endodermal nerve net in a radial canal of *Stomotoca* labeled with anti-tubulin antibody. (author, unpublished).

of epithelial cells, and the latter are connected by gap junctions to epithelia known to be excitable, and may therefore themselves be excitable. No one has managed to record simultaneously from a neuron and its epithelial sheath cell while excitatory or inhibitory transmission is occurring, so how the process works remains a mystery.

Work on *Aglantha* has revealed instances of what may represent 'cross-talk' between functionally distinct neuronal subsets as well as between nerves and adjacent epithelia (Mackie & Meech, 1995). For example, the conduction velocity of impulses in certain neuronal pathways varies according to whether or not other nerve groups are simultaneously active ('piggyback effect'). Conduction velocity can also increase with repetitive stimulation even when no other nerve groups are active. This could be explained in terms of ion channel kinetics but it is also possible that the surrounding epithelial cells hold a capacitative charge which assists forward flow of action currents in the neurons. The epithelial depolarizations known as 'slow waves' (W events) in *Aglantha* appear to reflect electrical activity of ensheathed neurons. Further work on these elusive phenomena may help to explain how epithelio-neural transition steps occur, and whether they are chemically or electrically mediated.

I began by asking where we go from here. The directions I have mentioned reflect my own interests. Other people will have different priorities. For instance, the ionic basis of electrical impulses conducted by epithelia and the properties of the channels responsible are very poorly understood. The neurotransmitters acting in neuro-epithelial transmission are not understood at all. Some of the effector responses spread by epithelial conduction would well repay further investigation, in particular the opacification, luminescent, and secretory responses described for *Hippopodius*. Finally, the complete absence of excitable epithelia in cnidarians other than hydrozoans continues to be surprising, given the many good uses that hydrozoans have found for epithelial conduction. To explain the discrepancy by saying it is because the other groups lack gap junctions merely pushes the puzzle back a step – why do they lack gap junctions? There is now evidence for both intercellular coupling and connexin-like protein in at least one anthozoan (Germain & Anctil, 1996, see also discussion in Satterlie, 2002). There is no shortage of interesting questions and plenty of room for new recruits to help answer them.

Acknowledgments

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Impulse conduction in the jellyfish *Aglantha digitale*

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Key words: T-type Ca^{2+} channel, TTX-insensitive Na^{+} channel, voltage-clamp, macropatch, K^{+} channel clusters, jellyfish swimming

Abstract

In the jellyfish *Aglantha digitale* two forms of swimming arise from two separate propagating axonal impulses: a fast, overshooting action potential that depends on TTX-resistant Na^{+} channels, and a low-amplitude spike that depends on T-type Ca^{2+} channels. While the Na^{+} action potential is propagated simply and without distortion, the shape of the Ca^{2+} spike depends on the past history of the axon; it is processed as well as propagated. Patch- and voltage-clamp experiments show how three classes of K^{+} channels contribute to this apparently unique system. A dual Na^{+}/Ca^{2+} impulse mechanism may increase the bandwidth of an axonal line of communication but it also places restrictions on the form of the synaptic input needed for spike initiation.

Introduction

It is clear that the major ion channel classes arose well before the appearance of nerves and muscles. There are voltage-gated K channels in the bacterium *Aeropyrum pernix* (Ruta et al., 2003), Na^{+}/Ca^{2+}-dependent action potentials in the heliozoan *Actinocoryne contractilis* (Febvre-Chevalier et al., 1986) and Ca^{2+}-dependent action potentials in *Paramecium caudatum* (Naitoh & Eckert, 1968) and in *Rhabdocalyptus dawsoni*, the glass sponge (Leys et al., 1999). In the first nervous systems these different channels come together to co-ordinate complex behaviour. The following is a short account of some of the properties of the five classes of ion channel found in the motor axons of the jellyfish *Aglantha digitale*. We have selected *Aglantha* for study because its behaviour is simple enough to analyse, yet complex enough to be interesting. As Gladfelter (1973) first observed, it is capable of two forms of swimming, as well as elaborate feeding movements. The aim here is to relate channel function directly to the animal's behaviour and to discuss what constraints might limit the utility of different ion channel classes. *Aglantha's* dual forms of swimming arise from two separate propagating axonal impulses, but this increase in bandwidth may place restrictions on the form of the synaptic input required for spike initiation.

Aglantha digitale

George Mackie and Claudia Mills have studied the behaviour of *Aglantha* during dives in the submersible *Pisces IV* (Mackie & Mills, 1983; Mackie, 1985). They record having seen large numbers around the northwestern coasts of North America at depths of about 200 m. They watched them escape contact with crab larvae, amphipods, and copepods. The form of escape that *Aglantha* exhibits is displayed at http://www.bris.ac.uk/Depts/Physiology/Staff/RWM/RWMWork/rwm.html. The fast, jet-propelled swimming movement (Donaldson et al., 1980) is, as far as we know, unique. Most jellyfish are severely limited in their defence repertoire. They crumple when they come up against anything unpleasant but a full-blooded

escape response seems well beyond them. Their musculature is a simple myoepithelium, so how does *Aglantha*'*s* more complex behaviour come about?

In spite of its simple anatomy, *Aglantha* is not only able to escape-swim but can also swim slowly and rhythmically like other jellyfish. During its regular 'fishing' cycles, *Aglantha* swims upward with its tentacles contracted; it then stops swimming and feeds with its tentacles extended while sinking under the influence of gravity (Mackie, 1980). The upward orientation during the swimming phase depends on input from statocysts at the base of the animal, and 'righting' movements during the sequence of swims bring the bell into an upright position. Each contraction propels the animal about one body length, and a swimming sequence consisting of about 30 contractions lasts about a minute.

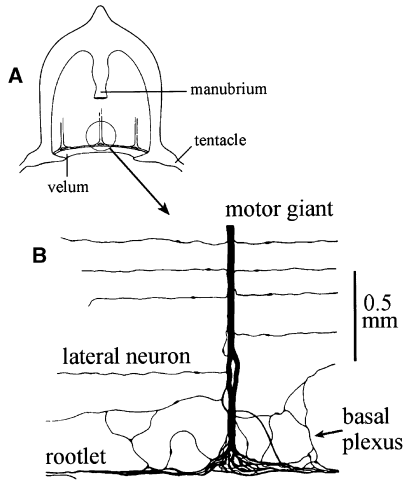

Figure 1. (A) Saggital section of *Aglantha digitale* (bell diameter in A ~5 mm) showing (B) the location of motor giant axons and the rootlet system. (Adapted from Weber et al., 1982.)

Anatomy of Aglantha

The epithelium on the inner surface of the bell-shaped body wall consists of electrically-coupled myoepithelial cells, each with a cell body and an elongated muscle tail. Lying on the myoepithelium, and making direct synaptic contact with it, are eight giant motor axons and a lattice of lateral motor neurons (Fig. 1). The anatomical relationship between the elements of the nervous system has been established from both Lucifer Yellow and carboxyfluorescein injections (Weber et al., 1982; Mackie & Meech, 2000) combined with anti-FRMFamide and anti-tubulin fluoresence microscopy (Mackie et al., 1985; Mackie & Meech, 2000).

The giant motor axons are synaptically connected through multiple rootlet neurons that run out from the bases of the motor axons and contact those of the neighbouring giant axons on either side. These rootlet neurons coordinate the escape-swim response elicited by tactile stimulation within the bell of the jellyfish and are part of an inner nerve ring that fulfills the role of a central ganglion (Mackie & Meech, 1995a, b, 2000). Within this inner nerve ring are numerous small nerve cells, some of which act as pacemakers for slow swimming.

At the base of the bell in a separate nerve ring outside the mesoglea is a ring giant axon. This coordinates the activity of the giant motor axons during an escape swim when a motor spike arises from activation of vibration-sensitive hair cells at the base of the tentacles (Arkett et al., 1988).

To complete the picture, a bundle of small nerves that run alongside each motor giant axon conducts action potentials from the margin to the manubrium, or mouth, of the jellyfish (Mackie et al., 1985). The information provided enables the manubrium to bend laterally towards captured prey located at the margin. Impulses are also conducted in strands of excitable epithelium running within the radial canals that lie alongside the giant motor axons (Mackie et al., 2003). These impulses inhibit the pacemaker neurons that generate the slow synaptic potentials that initiate each slow swim. They ensure that swimming is inhibited during feeding.

Escape-swim action potential and slow swim spike

Alan Roberts and George Mackie were the first to show that the most likely contenders for the escape response pathway, the eight giant motor axons, had rapidly propagating Na^+ spikes (Roberts & Mackie, 1980). When examined under the electron microscope, sections of the synaptic contacts between the motor axons and their surrounding myoepithelial cells showed large synaptic vesicles (100–150 nm diameter) and a synaptic cleft of 15–20 nm (Singla, 1978). We could confirm the presence of chemical transmission by simultaneous

recording from intracellular sites in the axon and the nearby myoepithelium (Kerfoot et al., 1985). At 10 °C, the delay between the peak of the action potential and the onset of the sharply rising post-synaptic depolarisation was 0.7 ms – too long for electrical transmission.

Identification of the nerve or nerves responsible for slow swimming proved more difficult. The small axons that run alongside the motor giant were an obvious target and yet even with them cut we could still observe slow swimming movements. This observation meant that the giant axon *had* to be implicated in slow swimming although *how* was not obvious, until we injected current directly into the axon with an intracellular micropipette (Mackie & Meech, 1985). We then discovered that we could elicit all-or-nothing low-amplitude spikes as well as overshooting action potentials in the same axon. Because the low-amplitude spikes were blocked with 7 mM manganese in the seawater we concluded they were likely to be Ca^{2+} based. Llinós & Yarom (1981) have described low-amplitude Ca^{2+} spikes in mammalian central neurons but each spike sets off an overshooting Na^{+} action potential and it is not clear whether the two signals can propagate independently.

What appears to happen in *Aglantha* is that the Ca^{2+} spike propagates along the axon keeping just under the threshold of the Na^{+}-dependent action potential. The resting potential in these axons is about −70 mV; the Na^{+}-dependent action potential has a threshold near −30 mV and so there is a 40 mV window into which the Ca^{2+} spike just fits. This enables the motor axons to propagate two entirely separate signals; the escape-swim Na^{+}-dependent action potential and the Ca^{+} spike responsible for slow swimming. But how is it that the amplitude of the Ca^{2+} spike is limited to this 40 mV window? To solve this problem we needed to study the axon membrane under voltage-clamp.

Voltage-clamp experiments

The difficulty with studying *Aglantha* axons under voltage-clamp is that the axon diameter (40 μm) is too small to insert longitudinal wire electrodes and too long (up to 20 mm) to obtain a satisfactory voltage-clamp with micropipettes. Fortunately, however, we found we could shorten the axon to about 0.5 mm using a sharp micropipette as a scalpel. The shortened fragment would reseal at each end and we were then able to obtain a passable space-clamp with two intracellular micropipettes.

A series of voltage-clamp experiments conducted in Na^{+}-free saline showed that depolarising commands close to the resting potential activated a T-type Ca^{2+} conductance (Meech & Mackie, 1993a). The amplitude of the Ca^{2+} current was steeply dependent on the holding potential so that if the membrane was held at −51 mV, the inward current was abolished entirely. Inactivation was fully removed only at membrane potentials more negative than −70 mV.

With a step change in membrane potential from −65 to −33 mV (i.e. near the peak of the Ca^{+} spike), the outward current turned on so rapidly that the inward current was concealed completely (Fig. 2). *In vivo* this would have the effect of limiting the amplitude of the spike and keeping it below the threshold of the escape-swim action potential. Separation of the two impulses in this way amounts to a division of the signalling process into low and high current systems.

Ionic currents recorded using in situ *patch-clamp technique*

We were unable to study the Na^{+} channel activation with the two-micropipette voltage-clamp because even in shortened axons, depolarising commands in normal sea water elicited such a large inward current that the axon escaped from control and the membrane potential changed regeneratively. Instead we have used *in situ* patch-clamp recordings to measure currents through small, isolated clusters of ion channels (Meech & Mackie, 1993b, 1995). Once we had cut the myoepithelium and exposed the motor axon, it was relatively easy to obtain a high resistance seal between the patch pipette and the axon membrane so that we were able to record the membrane currents without further treatment.

The currents recorded depend on the amount of cell membrane drawn into the patch pipette but with conventional sized pipettes (~1 μm tip OD) the membrane patch was found to contain as many as 200 K^{+} channels (Meech & Mackie, 1993b). There were at least three classes with 'fast,' 'slow,' and 'intermediate' kinetics, each class being

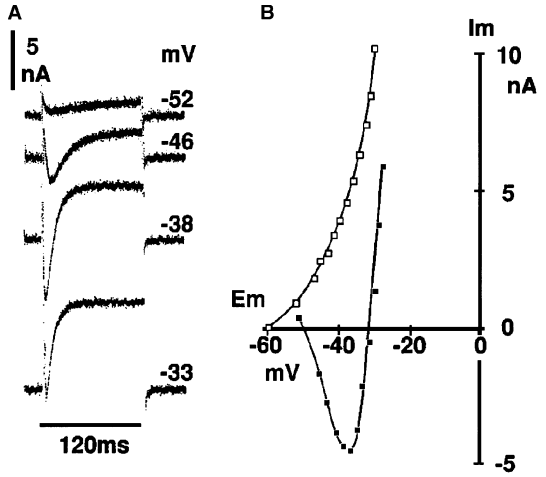

Figure 2. Voltage clamp of axon shortened to 800 μm and bathed in Na^+-free seawater substitute containing (in mM): 430 choline chloride, 10 KCl, 50 tetraethylammonium chloride, 10 $CaCl_2$, 50 $MgCl_2$, and 10 Tris–HCl (tris(hydroxymethyl)aminomethane-HCl; pH 7.9). Conventional voltage-clamp circuitry using thin walled glass micropipettes filled with 3 M KCl (resistance ~10 MΩ) was used. (A) Axon currents at different command potentials; interval between commands 60 s. Current records filtered at 1 kHz (corner frequency, 8 pole Bessel filter). (B) Axon current (I_m, ordinate) at different membrane potentials (E_m, abscissa) during a command step. Peak (filled symbols) and maintained (at 90 ms, open symbols) currents are shown. Data from same preparation as A. Axon resting potential −71 mV; holding potential −70 mV; temperature 10 °C (from Meech & Mackie, 1993a).

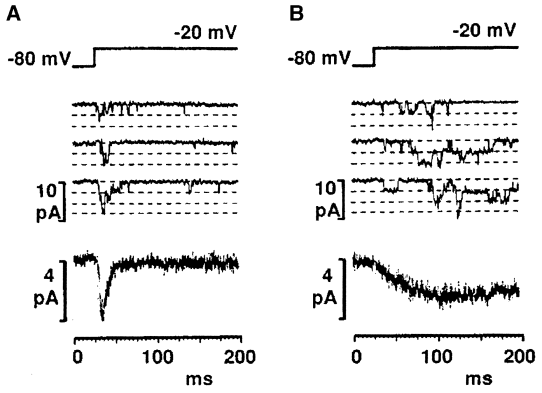

Figure 3. Channel currents recorded in potassium solution using the 'tight' seal patch-clamp technique; axon-attached configuration. Patch pipette filling solution (mM): 500 KC1, 50 $MgCl_2$, 10 $CaCl_2$, 10 Tris–HCl (pH 7.8). Axon resting potential taken to be −70 mV. Data from patches with slow, A, and fast, B, kinetics; all records were corrected for capacity and leakage currents. Each membrane patch was held at a membrane potential 10 mV more negative than resting, then depolarised by 60 mV. Calculated change in intracellular voltage is shown at the top of each series of traces. Because each patch contained three or more channels, many records showed multiple simultaneous openings. Currents flowing in the open state are shown by the dashed lines. An ensemble current average consisting of 48, A, and 79, B, consecutive records is shown below. Interval between pulses is 2.5 s, A, or 12 s, B (from Meech & Mackie, 1993b).

organised in uniform clusters. All three channels showed inactivation, all three recovered from the inactivated state within 10–20 s, and all three had the same unitary conductance. Two of the three channel types are shown in Fig. 3. In the records shown, the membrane patch (fortuitously) contained a single class of channel; in other records the patches contained combinations of the three types (Meech & Mackie, 1993b). The channel clusters appear to form a mosaic over the axon surface so that it is possible to record from different combinations of channel type depending on the location of the recording pipette.

The infrequency with which we obtained patches with a significant inward current led us to suppose that Na^+ and Ca^{2+} selective channels were present in well-separated 'hot spots'. So, to increase the likelihood of recording an inward current, we used recording patch pipettes with enlarged tip diameters (4–6 μm OD). Pipettes were filled with seawater and had a relatively low resistance (~450 kΩ) so that as long as the seal was better than about 5 MΩ, errors in current measurement were small (Stühmer et al., 1983).

Large patches of axon surface were drawn onto the end of the pipette by gentle suction (Meech & Mackie, 1995). Figure 4A shows the Na^+ current recorded from one such macropatch after light suction. With further suction, more surface membrane was drawn into the pipette and a Ca^{2+} current became evident with the same voltage command (Fig. 4B). The Ca^{2+} current recorded from such 'hot spots' had the characteristics of a T-type channel.

T-type Ca^{2+} currents in Aglantha

As expected the T-type currents were highly sensitive to small changes in holding potential. They were fully inactivated with the holding potential at −50 mV. Inactivation of 50% occurred with a holding potential of −58 mV, somewhat more

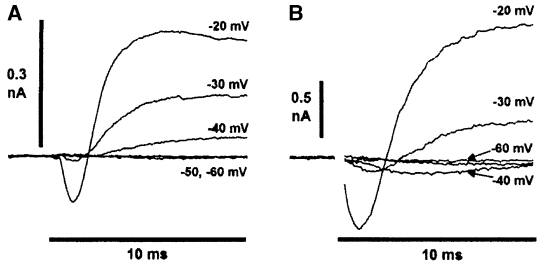

Figure 4. *In situ* patch pipette recordings from *Aglantha* motor giant axon using the 'loose' patch clamp technique (Stühmer et al., 1983). Membrane potential during the command pulse is at the right of each set of records. Axon resting potential taken to be −70 mV. (A) Currents from membrane patch in response to step change in patch potential. Seal resistance, 42 MΩ. (B) Membrane currents from same patch after further suction. Seal resistance, 109 MΩ. Onset of the step command corresponds to the left edge of the time scale bar. Residual capacitive currents at onset of step command are blanked out of record. Patch pipette filled with normal seawater. Pipette tip, 4 μm OD; Pipette resistance, 437 kΩ; holding potential, −90 mV; temperature, 11 °C (from Meech & Mackie, 1995).

positive than in striated muscle cells from the jellyfish *Polyorchis penicillatus* (−80 mV; Lin & Spencer, 2001) and in most mammalian cells (−70 to −80 mV), but comparable to T-type channels in cardiac ventricular myocytes. Inactivation followed a rapid time course and so too did the process of recovery. At −70 mV currents recovered more rapidly ($\tau = 30$ ms) than those in mammalian cells (τ usually 200–300 ms) but in similar fashion to T-currents in *Polyorchis* ($\tau = 51.7$ ms; Lin & Spencer, 2001). In *Aglantha,* the T-type current recovered from inactivation more rapidly than any of the axonal K^+ currents.

In mammalian cells T-channels are usually found together with high voltage-activated Ca^{2+} channels and it is thought that the T-currents contribute to events near threshold or to pacemaker type activity. T-currents are often so small that it is not clear whether they can actually generate the increase in internal Ca^+ required for synaptic transmission. We have been unable to find any other class of Ca^{2+} channel in *Aglantha* (on either upper or lower surfaces of the motor axon; Meech & Mackie, 1995) and it may be that Ca^{2+} influx through T-type channels sets off Ca^{2+}-induced Ca^{2+} release under the axon membrane as may occur in the striated muscle cells of *Polyorchis* (Lin & Spencer, 2001).

Slowly inactivating Na^+ currents in Aglantha

In order to record the time course of the inward Na^+ current, we used 5 μm diameter patch pipettes filled with an artificial seawater containing 10% Na^+ and 50 mM tetraethylammonium ions (TEA). The low Na^+ solution reduced the amplitude of the inward current and prevented a 'patch spike,' TEA blocked the fast and slow K^+ channels, and the intermediate K^+ channels were largely inactivated by holding the membrane potential at −45 mV (Meech & Mackie, 1995). When we isolated the Na^+ currents from the rapidly activating K^+ currents we found that activation peaked at 1.4 ms at 10 °C and that inactivation proceeded at a slow rate ($\tau \sim 12.5$ ms at +10 mV). Half-recovery from inactivation was also slow (∼200 ms). As we already knew that the escape-swim spike was insensitive to tetrodotoxin, it seemed possible that the Na^+ channels were similar to those present in mammalian cardiac membranes and spinal sensory neurons (Kostyuk et al., 1981; Campbell, 1992). Cardiac-like Na^+ currents with similar properties have also been recorded in the motor neurons of the jellyfish *Polyorchis* (Grigoriev et al., 1996). The α subunit of the Na^+ channel is known to determine its main electrophysiological and pharmacological properties (Catterall, 1992) but the β1 subunit promotes rapid inactivation and recovery (Isom et al., 1992; Wallner et al., 1993; Nuss et al., 1995). It may be that the β1 subunit is absent in *Aglantha* motor axons.

Synaptic input into the giant motor axon

Slow swimming in other jellyfish species depends on large pacemaker neurons in the nerve ring at the base of the animal (Anderson & Mackie, 1977; Spencer, 1978). In *Aglantha* these neurons form a pacemaker system (Mackie & Meech, 1995a, b) that is modulated by nitric oxide (Moroz et al., 2004). Stimulation of the pacemaker system sets off a low-amplitude, slowly rising, post-synaptic potential (peak amplitude at ∼7 ms) in each of the eight giant motor axons (Meech & Mackie, 1995). The threshold for the production of Ca^{2+} spikes by these slow events is about −51 mV which is close to the voltage at which a net inward T-type Ca^+ current first appears during depolarising commands.

Escape swimming, on the other hand, appears to start with vibration-sensitive hair cells at the base of the tentacles (Arkett et al., 1988). These hair cells feed into the giant axon that runs round the nerve ring at the base of the animal – the ring giant. An impulse in the ring giant axon sets off an overshooting action potential in the giant motor axon via a large, fast rising (time to peak ~1 ms) post-synaptic potential. The threshold for the initiation of the Na^+ spike is about −32 mV. Injecting current through one micropipette varies the axon membrane potential as measured with a second micropipette a short distance away. Extrapolation of the linear relationship between membrane potential and the post-synaptic potential amplitude shows the reversal potential to be near 0 mV. Thus the synaptic input behaves in a manner expected for a chemical synapse.

Escape swimming is elicited by stimulating the apex of the animal as well as the base. In this case the excitation proceeds via one motor axon and is conducted to others by way of connections between rootlet neurons. The post-synaptic potential recorded at the base of the motor axon is similar in its amplitude and rise time to that provided by the ring giant axon but arises at an electrical rather than a chemical junction. In this case experimental manipulation of the membrane potential of the motor axon has little effect on the amplitude of the event recorded in the axon (Meech & Mackie, 2001).

Ca^{2+} spikes during slow swimming sequence

When recording from the motor axon, we found that endogenously generated slow swim spikes were variable in shape, the first of a series having a shorter duration and a pronounced undershoot (Fig. 5; Meech & Mackie, 1993a). The spike undershoot disappears because a significant proportion of the K^+ channel population remains inactivated for several seconds. As a consequence later spikes tend to have a longer duration and produce stronger responses in the myoepithelium. Because of its undershoot, the first spike of the series (important for righting the animal as it begins to swim upward again) produces one of the weakest responses. Note that when the spikes are closest together there is no myoepithelial response, presumably because the inactivated Ca^{2+} channels

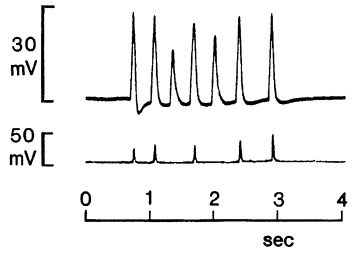

Figure 5. Sequence of spontaneous low-threshold calcium spikes (upper) at a recording site 5.5 mm from ring giant axon (Fig. 1) with corresponding post-synaptic electrical events in adjacent muscle cells (lower). Intracellular recording micropipettes filled with 3 M KCl (resistance, 8–12 MΩ). Mechanical disturbance associated with muscle contraction was reduced by bathing the preparation in high-Mg^{2+} seawater (Mg^{2+} 65 mM) (from Meech & Mackie, 1993a).

are not yet fully recovered and so the Ca^{2+} influx is too small to release synaptic transmitter.

Discussion

Given that the major ion channel classes evolved in single celled organisms, it seems likely that the metazoan state imposes additional constraints on channel properties and organisation. The evolution of impulse propagation along tubes and through epithelia has provided a means to develop complex behaviour. Non-decremental impulse propagation places stringent demands on the contributing ion channels. *Paramecium*-like graded spikes (Naitoh & Eckert, 1968) studied in the NEURON Simulation Environment (Hines & Carnevale, 1997) do not propagate in an axonal architecture. Travelling action potentials occur in the heliozoan *Actinocoryne* (Febvre-Chevalier et al., 1986) and the dinoflagellate *Noctiluca miliaris* (Eckert, 1965) but the distances are short (250–600 μm).

Propagating Ca^{2+}-dependent action potentials are present in some higher plants (lijima & Sibaoka, 1985) and in the sponge *Rhabdocalyptus* (Leys et al., 1999), but impulse conduction in most nerve axons is Na^+-dependent as is most epithelial conduction (Mackie, 1976; Grigoriev & Spencer, 1995; but see Josephson, 1985). A Ca^{2+}-current-based solution is responsible for signalling in nematodes (Bargmann, 1998), but it is a largely analogue mechanism (Davis & Stretton, 1996).

Hille (2001) suggests that rapid all-or-nothing conduction in nerve axons is based on the Na^+-channel because intracellular Na^+ has a lower toxicity than intracellular Ca^{2+}. An alternative explanation is that Ca^{2+} channels are markedly temperature sensitive (Byerly et al., 1984). The slow swim spike in *Aglantha* may avoid this problem because T-type channels are significantly less temperature sensitive than other Ca^{2+} channels (Nobile et al., 1990). Q_{10} values for T-type channel kinetics are generally close to those of voltage-gated Na^+ or K^+ channels (range: 1.7–3.5; Coulter et al., 1989; e.g. Nobile et al., 1990; Takahashi et al., 1991) whereas other Ca^{2+} channels have kinetics with Q_{10} values of about 5 (e.g. Byerly et al., 1984; Delbono & Stefani, 1993; McMorn et al., 1998).

The temperature sensitivity of L-type Ca^{2+} channels may be connected to their modulation by phosphorylation (e.g. Kostyuk & Verkhratsky, 1995). This distinguishes them from other voltage-gated channels (including T-type Ca^+ channels) and it relates to Ca^{2+}'s role as an intracellular messenger. Generally nerve axons transmit impulses without distortion, the processing of information being confined to the cell body or the nerve terminal, and it may be for this reason that Na^+-dependent action potentials are suited to axonal propagation.

It is striking that T-type channel impulses have been described only in *Aglantha* motor axons. It is true that a similar spike is present in mammalian central neurons (Llinás & Yarom, 1981) but it is not clear that it ever occurs separated from an accompanying Na^+-dependent action potential. The explanation for its limited distribution may be that its repolarising phase involves the activation of a complex set of K^+ channels whose inactivation can modify the shape of the spike. In *Aglantha* the motor axon behaves like an extended nerve terminal because synaptic contacts with the underlying myoepithelium are distributed along its entire length. While the Na^+ spike is propagated simply and without distortion, the Ca^{2+} spike is dependent on the past history of the axon; it is processed as well as propagated. This dual Na^+/Ca^{2+} system of impulses appears to be unique to *Aglantha*. On the face of it, it seems an ideal way in which to increase the bandwidth of any line of communication. So what is the problem? Most probably it lies in the restrictions imposed on the synaptic input to the axon. Because the Na^+-dependent action potential has such a high threshold, the synaptic input is required to be stereotypically large and fast rising. Consequently features normally associated with synaptic integration, such as summation, are excluded.

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Spasm behavior and the diffuse nerve-net in *Cassiopea xamachana* (Scyphozoa: Coelenterata)

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Key words: sub-umbrellar muscles, spasm behavior, diffuse nerve-net, *Cassiopea*

Abstract

A quick train of diffuse nerve-net (DNN) impulses is required to initiate a patterned flurry of strong contractions, the 'spasm,' of the striated muscles of the rhizostome scyphomedusan *Cassiopea xamachana*. A number of spasms are illustrated. Using this response as a tool, the connectivity of the DNN has been established. The occurrence and variability of the spasm is illustrated with an analysis of their occurrence in a typical, extended experiment. It is proposed that the spasm is an integral and essential component of the behavior of scyphomedusae, and that its control by the DNN exemplifies how complex behavior is generated.

Introduction

Jellyfish have attracted the interest of many biologists because they are conspicuous and active marine predators with a seemingly limited repertoire of behavior. The two main groups of medusae, the Scyphozoa and the Hydrozoa, are very different in their neurophysiology (for review, see Satterlie, 2002). The large jellyfish (scyphomedusae) have marginal nerve ganglia in the rhopalia[1] but no bundles of nerve fibers. Their two distinct nerve-nets, the giant fiber nerve-net (GFNN)[2] and the diffuse nerve-net (DNN), both consist of a feltwork of bipolar and multipolar nerve cells lying within the spaces of the ectodermal epitheliomuscular tissue. Functionally their only connection is at the ganglion. The subumbrellar striated swimming muscles are innervated by both nerve-nets. In some species a single nerve impulse in either leads to a muscle contraction, larger in the case of the GFNN pulse, smaller to the DNN pulse; however, in *Cassiopea* while a GFNN pulse produces a muscle contraction graded in intensity by previous activity, a DNN pulse only 'facilitates' or increases the strength of the muscle's response to a GFNN pulse but does not cause a muscle contraction by itself. In this system of neuromuscular control the characteristic of the muscle response, its strength and duration and the precise timing necessary to shape the water jet, is determined by the neuromuscular delay time and the muscle facilitation. It seems likely that the neuromuscular delay at least is a property of the muscle cells, since a single GFNN impulse sweeping over the subumbrellar striated musculature leads to very different delays in the different muscle blocks. Muscle contractions are not regulated by the frequency of nerve impulses nor by recruitment of more fibers. For details see the review by Passano (1982). The animals show no evidence of experiencing pain and elaborate vivisection is always tolerated (Romanes, 1885; Mayer, 1910).

[1] Usually eight rhopalia occur in scyphomedusans; however in *C. xamachana* the number is variable and greater, usually around 16.

[2] The original term of Horridge (see Bullock & Horridge, 1965). It is also called the 'motor nerve-net' by many authors (Satterlie, 2002).

Nerve impulses from both nerve-nets have been recorded but electrical signals are much harder to detect in scyphomedusae than in hydromedusae, due in the latter both to the presence of bundles of axons and of the signs of electrical synapses. In addition, there is no epithelial conduction in scyphozoans. For them it is much simpler to monitor long term behavior by recording the muscle contractions directly.

A primary goal of this project is to understand the role of the nervous system in generating the behavior of these animals. With the discovery described here of a specific qualitative measure of DNN activity, the 'spasm,' it has been possible to demonstrate the functional connectivity of the DNN throughout the ectoderm of the exumbrella, subumbrella and oral arm structures of the rhizostome scyphomedusae, and thus increase our understanding of the contributions of this part of the nervous system.

Materials and methods

The bottom-living upsidedown jellyfish *Cassiopea xamachana* (Bigelow, 1892) was used exclusively in this study. Animals were collected commercially from Big Pine Key, FL, and shipped by overnight airfreight to the laboratory. The preparation was pinned in the animal's normal 'oral-arms-up' position with fine insect pins (0.3 mm diameter) to a 'Sylgard' coated platform covered with 1.3 l of 'conditioned' tank sea water. Muscle contractions were recorded with mechanoelectrical transducers connected with very fine Ag wire (0.03 mm diameter) above the deep recording dish to a hook made of Pt-Ir wire (0.125 mm diameter) through the subumbrella muscle sheet. Routinely, preparations were monitored for periods of more than 24 h in the dark. The entire recording chamber was in a Faraday cage on a vibration-damping table. Polygraph recording was conventional, but muscle contractions were recorded with pen deflection either up or down.

Commonly a single animal was made into two separated halves, each reduced by extirpation to having two or three rhopalia, and with a DNN-conducting connection but no GFNN conducting pathway. The DNN conducting bridge was made in one of two ways. The connection pathway could go from one subumbrellar region up into the collar of the oral apparatus and then return to the other piece's subumbrella, as shown in Figure 1. An alternative DNN-only pathway could be created looping around the margin and up on to the exumbrella before going back around the margin again to the other block of subumbrella. In this report these are termed 'conjoint preparations.'

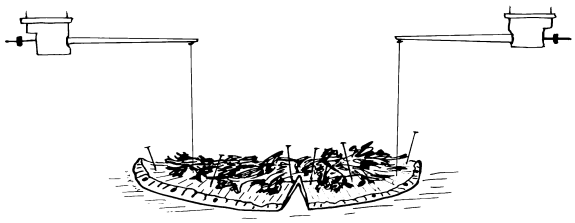

Figure 1. Sketch of the 'conjoint preparation.' A V cut is made through the umbrella into the collar region that supports the oral arms, in the middle of a half animal preparation, blocking all GFNN conduction between the two pieces. Spontaneous subumbrellar muscle contractions are recorded with two sensitive transducers.

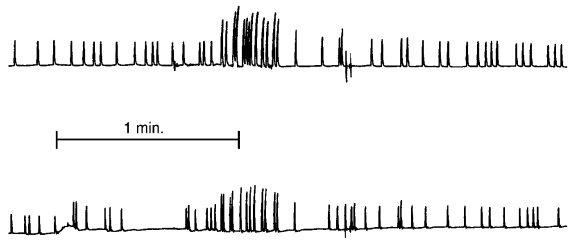

Figure 2. A polygraph record of muscle contractions in a conjoint preparation, showing spasms induced by touch stimulation.

In either case, the successfully created DNN-only conduction pathway was immediately demonstrated by the separate unconnected spontaneous swimming contractions of the two pieces and the common response of a spasm initiated in both halves to gentle touch stimulation with a polyethylene probe made from a suction electrode (Fig. 2) on any piece of DNN-conducting tissue. It should be noted that the individual contractions that make up the spasm are not coincident.

The term 'spasm' is used here[3] to refer to a quick series of powerful contractions of the striated muscles of the subumbrella of the *Cassiopea*.

[3] Romanes (1885) used the term 'spasm' for behavior in hydromedusae to refer to what subsequent authors have called 'crumpling.'

These muscles are homologous to those responsible for swimming in the usual pelagic scyphomedusae, but normally in this species that lies on the bottom they are used to produce the languid body pulsations, the pulse, that bring food to the mouths on the oral apparatus. The frequency of these pulsations depends on the size of the animal; newly released ephyrae pulse at 3 per s and show regular swim bouts. The larger medusae collected (200 mm diameter) may pulse at 1 per 10 s while lying on the substrate.

In its natural environment, a *Cassiopea* may rise in the water column, turn over, and swim, as for instance when it is being uncovered by a receding tide. In the laboratory as many as 10% of the newly collected animals in prime condition may swim in the tank, but this behavior gradually disappears after a week or so.

Results

Individual spasms

A train of 4–6 electric shocks (8 v, 2 ms) delivered at a frequency of 3 per s on a block of tissue isolated from any direct GFNN conduction pathway can induce spasms just as the touch with a probe described above. This induced spasm (Fig. 3, left) can be compared to a spontaneous spasm that occurred 9 min later (Fig. 3, right). Induced spasms generally are neither as powerful nor as long lasting as spontaneous spasms but the ability to induce spasms, a distinctive and recognizable response, gives us a tool to use to map DNN connectivity throughout the animal.

Frequently two conjoint preparations were studied simultaneously in two separate 1.3 l dishes. Individual spasms were almost never found to

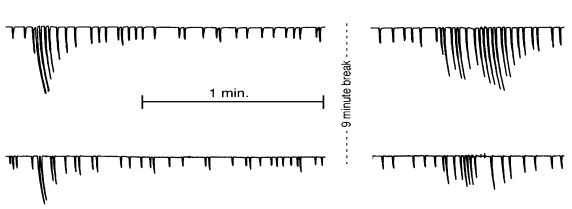

Figure 3. A polygraph record of another conjoint preparation, with induced spasms following electrical stimulation (left) and, 9 min later, spontaneous spasms (right).

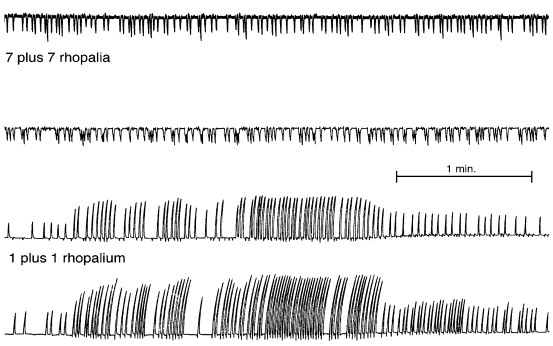

Figure 4. A polygraph record of two conjoint preparations made from medusae with 14 (upper pair) and two (lower pair) rhopalia respectively. The upper pair shows an enhanced pulse rate; the lower pair shows spontaneous spasms.

occur in these preparations simultaneously. In ongoing investigation of the natural occurrence of spasms in pieces with unequal numbers of rhopalia, two pieces of equal size were made with 14 rhopalia (Fig. 4, upper pair; Fig. 5, top & middle) and two rhopalia (Fig. 4, lower pair; Fig. 5, bottom record), by excising most rhopalia in the latter preparation. The piece with the much larger number of rhopalia shows a greater frequency of pulsations and a greater intensity and duration of the spasms but the frequency of spontaneous spasms did not vary significantly. These spontaneous spasms shown in Figures 4 and 5 occurred 76 min apart.

Spontaneous spasms vary widely in the number and frequency of the individual contractions that make them up. This may reflect the variability of the immediately preceding spasm trigger events in

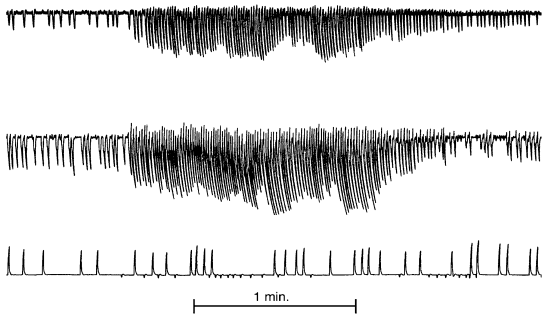

Figure 5. The same preparation as Figure 4, 76 min later. Spontaneous spasms in preparation with 14 rhopalia. The lowest of the polygraph records was omitted from the figure (due to an ink failure) but that piece's pulse contractions are signaled by the small blips on the other record from that preparation.

the DNN, but must also involve the state of the GFNN pacemaker(s) that directly generate the spasm as well as the body pulsations. When spasms are recorded from two preparations connected by a DNN-only conducting pathway, the spasms differ. If one half has been considerably more active than the other, the spasm triggered in the more active piece usually has more individual contractions (Figs 4 and 5; see also Fig. 2). The individual spasms may also be of longer duration. This suggests that the parameters of spasms are determined, at least in part, by the state of the 'spasm response mechanism' rather than by the form of the trigger.

Sometimes a spasm is briefly interrupted and then resumes (Fig. 4, lower pair). Occasionally short spasms occur repeatedly; in the example illustrated in Figure 6 very similar spasms reoccurred twice, with about 75 s separating each spasm. In both of these examples the character of the spasm of each piece is similar to the character of the ongoing pulse output of the GFNN pacemaker(s); if the latter is rapid and strong, so is the spasm.

If the DNN pathway is cut, subsequent spasms do not occur in synchrony in the two blocks of subumbrellar tissue. This is illustrated in Figure 7. In extended experiments over several days the severed connection may be reestablished by tissue regeneration after periods of intermittent conduction.

Occasionally, and especially when the GFNN pacemakers are not very active (i.e. when the pulse rate is slow), a spasm occurs on one side of the bridge while the other side shows strong pulses (due to the facilitation effect of the DNN activity on the muscles) but the frequency of the pulses is low. Sometimes there are transient small increases in amplitude of the pulse outputs of both pieces of

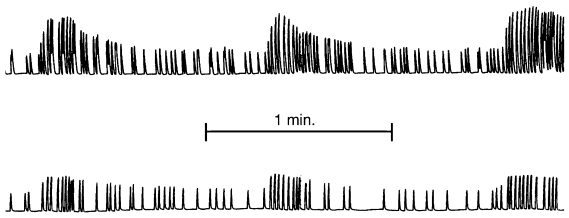

Figure 6. Three spontaneous quick spasm events in a conjoint preparation.

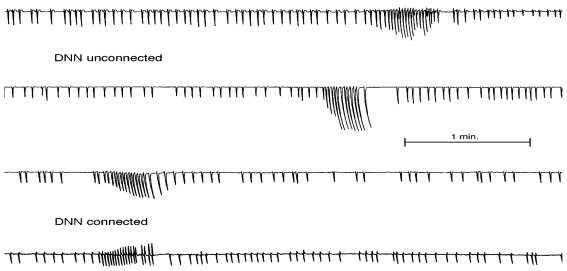

Figure 7. Two preparations made from a single animal and recorded from the same dish to show spontaneous spasm events. In the upper preparation the DNN-conducting bridge had been severed, resulting in uncoordinated spontaneous spasm events from each piece with three rhopalia. The lower paired records from a conjoint preparation with two rhopalia and one rhopalium show a coordinated event.

the conjoint preparation but no spasm. This can be explained as evidence of DNN activity that is below the threshold of the spasm trigger.

Spasm occurrences over time

The occurrence of a spasm is occasional and sporadic. And although I have encountered exceptions, the general rule is that they first occur at least 2 h after the preparation is set up, and this initial delay can be much longer. I have no explanation to offer for this delay.

As an example: an experiment was begun on February 25th, using two halves of a jellyfish collected in Florida February 4th and shipped by air freight to the laboratory. Each half, pinned to a platform covered with sea water was made into the conjoint preparation as described above. In 'I/II' the two parts are connected with a DNN-conducting strip of ectoderm; in 'III' and 'IV' the connecting strip was scored with a scalpel cut to break the DNN continuity and leave two independent pieces. Subumbrellar muscle activity was then recorded for a period of 30 h with nearly 400 recorded spasms, an unusual result both in their number and their relative regularity.

The first spasm occurred after a 2.4 h delay for I/II, also 2.4 h for III and only after 10.7 h for IV. Thereafter there occurred a total of 130 spasm-pairs for I/II, 85 spasms for III and 46 for IV in the remaining experimental time. Since the paired preparation I/II generated twice as many spasm events as the average of the separated pieces, and since each piece had three rhopalia, this might be

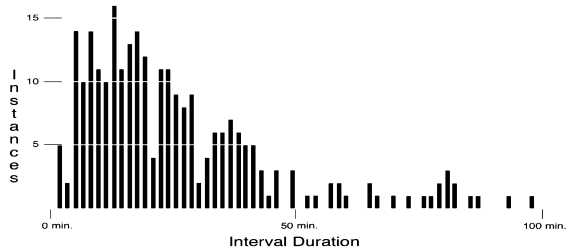

Figure 8. Time intervals between spasms. A single experiment with 391 spontaneous spasms in 31 h. See text.

seen to suggest that generation of the DNN events associated with the spasm originates at the rhopalial marginal ganglion.

The time between spasms varied between the three groups. As expected, the double piece connected by the DNN conducting pathway, termed here I/II, showed a shorter average time interval with less variability (12.2 min ± S. D. 8.8 min), than either of the single pieces (III = 19.3 ± 20.8 min; IV = 25.3 ± 14.9 min). Over all the total spasm intervals, shown in Figure 8, seem to be distributed normally but with an elongated tail for the occasional long time interval between spasms. The two longest outlying intervals are omitted from the graph.

Discussion

The first problem faced after the discovery of the spasm behavior was to rule out the possibility that it was triggered by some sort of laboratory artifact. Considerable care was exercised to shield the preparation from any sort of periodic vibration, etc., but the best proof of the normality of the events was the failure to experience simultaneous spasms with two preparations in the same recording dish. Furthermore the simultaneous spasms occurring in the two halves of a preparation joined only by a DNN-conducting tissue bridge were always decoupled when the bridge was severed. This finding leads to the second discovery: a DNN nerve impulse is required to trigger the spasm. Using electrical stimulation, it was determined that a brief train of at least three DNN impulses was needed to trigger this event.

While the work reported here demonstrates how it is possible to deliver experimentally a DNN pulse without an induced GFNN pulse, there is no reliable method to deliver a GFNN pulse without the probable generation of a DNN pulse as well. This is the problem with the classic work by Bullock (1943) that first demonstrated the phenomenon of facilitation in scyphomedusae, and it confounds the logic of the argument of Pantin & Vianna Dias (1952) as well. These authors had no control over the fact that stimuli given to the subumbrellar epithelium probably induced both GFNN and DNN responses. It is now known from experiments where activity in both nerve-nets was monitored that both GFNN and DNN impulses facilitate muscle contraction responses. These results obtained with tightly restrained animals (Passano,1965) supports the notion that the regular muscle contractions of the striated subumbrella muscles, referred to here as the 'pulse' contractions, are due to single GFNN pulses without any DNN involvement. The GFNN is as Horridge first formulated (see Bullock & Horridge, 1965), a rapid through-conducting dedicated motor control system for the pulse contractions of this primary effector. The single GFNN impulse also resets all the marginal ganglion pacemakers that are responsible for the regular pulse contractions. In other species of scyphomedusae such as *Cyanea capillata* either one or a pair of GFNN nerve impulses may trigger a single striated muscle contraction, if the second pulse occurs within the muscle absolute refractory period (ARP). The muscle ARP is two orders of magnitude longer than the ARP of the GFNN in *Cyanea*, thus explaining why the presence of pairs of GFNN pulses are not discernable by the appearance of elongated muscle contractions (Passano, 1982). The role of such pairs of GFNN pulses is not known.

What then of the other nerve-net? It is not, primarily, a sensory system; there are neither obvious DNN reflexes nor DNN sensory input into a brain. The sensory information from the light and gravity receptors does not go to the marginal ganglia via the DNN, but on local specialized pathways in the rhopalia. The DNN is the motor nerve-net for the scyphopolyp and ephyra (Horridge, 1956a) stages of development. A DNN pulse or a slow train of pulses controls the movements of the oral arms or of the tentacles (absent in *Cassiopea*). It augments the contraction strength of the swimming muscles as Horridge (1956b) first observed in the 'entrapped wave' preparation (reviewed in Mayer, 1910). It facilitates the marginal

ganglion pacemakers (Passano, 1965, 1982) after a characteristic delay. And, as it is shown here, a quick train of DNN pulses triggers the spasm, which may be a special case of its role on the pacemakers since the two responses share much in common.

I made use of the fact that a quick train of DNN pulses triggers a spasm to map the connectivity of this nerve-net. DNN pulses always pass around the margin of the umbrella, subumbrella to exumbrella, or vice versa. The DNN also extends throughout the oral apparatus of this order, the Rhizostomeae. It has not been determined whether the endodermal DNN is continuous with the ectodermal DNN (Passano & Passano, 1971).

Swimming is only a minor component of the biology of this semi-sessile jellyfish, but it is of major importance to many scyphomedusae. The complex biology of intermittent swimming was brought home with the observations of Mackie et al. (1981) of the behavior of large groups of *Aurelia* in the deep observation tank at Dalhousie University. Intermittent swimming may be interrupted with periods of 'fishing' or other prey-catching activities. The role of the DNN in triggering spasms in *Cassiopea* is one example of the way a simple conducting network can be programmed by evolution to produce complex behavioral events. Ultimately coelenterate neuroethologists must explain how such elaborate behavior can be generated by a two nerve-net system with marginal ganglia. An entirely different but no less important problem that the parallel evolution of the hydromedusae poses is the comparison of the control systems of these two groups of cnidarians with distinct swimming phases (Satterlie, 2002).

We have no evidence about where within the animal the DNN spasm trigger events normally arise. Since extirpation of most of the rhopalia does not significantly reduce the initiation of spasms it seems unlikely that these triggers arise there even though, paradoxically, their presence shapes the intensity and duration of the spasm. The possibility that secondary DNN pulses can be generated by spasm behavior also needs further investigation.

Acknowledgments

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Mechanoreception and synaptic transmission of hydrozoan nematocytes★

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Key words: Corynidae, *Hydra*, mechanoelectric transduction, hair cell, chemoreception, synaptic exocytosis

Abstract

Mechanoelectric transduction and its ultrastuctural basis were studied in the cnidocil apparatus of stenotele nematocytes of marine and freshwater Hydrozoa (*Capitata* and *Hydra*) as a paradigm for invertebrate hair cells with *concentric* hair bundles. The nematocytes respond to selective deflection of their cnidocil with phasic-tonic receptor currents and potentials, similar to vertebrate hair cells but without directional dependence of sensitivity. Ultrastructural studies and the use of monoclonal antibodies allowed correlating the mechanoelectric transduction with structural components of the hair bundle. Two other types of depolarising current and voltage changes in nematocytes are *postsynaptic*, as concluded from their ionic and pharmacological characteristics. One of these types is induced by mechanical stimulation of *distant* nematocytes and sensory hair cells. It is graded in amplitude and duration, but different from the presynaptic receptor potential. Adequate chemical stimulation of the stenoteles strongly increases the probability of discharge of their cnidocyst, if the chemical stimulus precedes the mechanical one. Simultaneously, the probability of synaptic signalling induced by mechanical stimulation is increased, reaching nearly 100%. The chemoreception of the phospholipids used could be localized in the shaft of the cnidocil, because of the water-insolubility of the stimulant. This chemical stimulation itself does not cause a receptor potential; its action is classified as a modulatory process. Electron microscopy of serial sections of the tentacular spheres of *Coryne* revealed synapses that are *efferent* to nematocytes and hair cells besides neurite–neurite synapses, each containing 3–10 clear and/or dense-core vesicles of 70–150 nm diameter. The only candidates to explain the *graded afferent* signal transmission of nematocytes and hair cells are regularly occurring cell contacts associated with 1(–4) clear vesicles of 160–1100 nm diameter. *Transient* fusion and *partial* depletion of *stationary* vesicles are discussed as mechanisms to reconcile functional and structural data of many cnidarian synapses.

Introduction

Hydrozoan nematocytes exhibit the configuration of mechanosensitive hair bundles that is characteristic for epithelial mechanosensory cells of most invertebrate phyla. This is the *concentric* hair bundle (Fig. 1b). In contrast to the multicellular ciliary cone of anthozoan nematocytes (Watson & Mire, 2004), the hair bundle of hydrozoan nematocytes, the cnidocil apparatus, is a product only of the nematocyte (Slautterback, 1967). The cnidocil apparatus closely resembles

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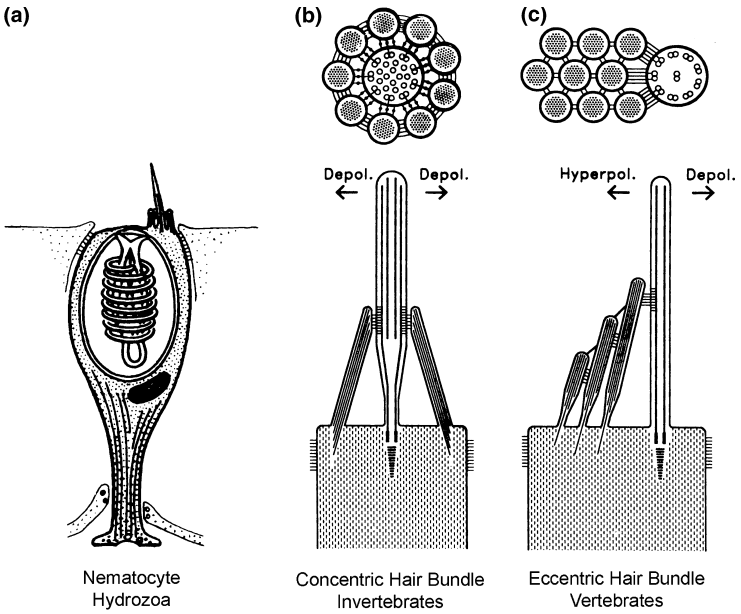

Figure 1. Schemes of (a) a hydrozoan nematocyte (in textbook resolution), (b) a concentric hair bundle of invertebrate hair cells (collar receptors), and (c) an eccentric hair bundle of vertebrates. In b and c bottom: longitudinal sections; top: cross sections at the level of membrane connections (composite section in c). Arrows indicate directions of stimulatory deflections for de- or hyperpolarizing receptor potentials (adapted from Thurm et al., 1998a, b).

the hair bundle of oldest mechanosensory hair cells (Tardent & Schmid, 1972; Golz & Thurm, 1994; Holtmann & Thurm, 2001b) and its basic organization is retained up to the lower chordates (Hüesker & Thurm, 1994). Hydrozoan nematocytes may be the best accessible paradigm to study concentric hair cells.

In several hydrozoa we found that nematocytes transmit afferent synaptic signals to other cells. This means, their mechanosensory transduction controls exocytotic processes not only at the apical side, i.e. the discharge of the cnidocyst, but also at the basolateral side, i.e. the release of transmitter, as in sensory cells. This raised the question of the structural basis of this output; afferent synapses had not been reported so far for hydrozoan nematocytes. The result challenges the concept of synapses of coelenterates.

Different from mechanosensory cells, exocytotic activity of nematocytes is known to be under additional control of a second, the chemical, modality (for *Hydra*: Lentz & Barrnett, 1962). The mode of interaction of mechanical and chemical modalities proved to be a revealing issue.

Material and methods

Our objects for structural as well as for physiological studies were freshwater *Hydra* (mostly *H. vulgaris*) and marine Corynidae (*Stauridiosarsia producta*, *Coryne tubulosa*, and *Dipurena reesi*). Transmission electron microscopy was optimized to stabilize and to stain cytoplasmatic and extracellular connecting structures and cytomembranes (Golz & Thurm, 1991). The noncontractile tentacular spheres of the capitate Corynidae allowed stable electrical recordings from nematocytes in their natural connectivity while the tentacle was isolated and held by a suction capillary (Brinkmann et al., 1996; Fig. 2). Tentacles of *Hydra*, isolated for electrical recordings, were immobilized by 1 μM HA-1077, a blocker of myosin phosphorylation (Lawonn, 1999). A glass probe that was moved in two dimensions by piezo-electric drivers and was opto-electronically controlled in feedback loops achieved mechanical stimulation of the individual cnidocil. Intracellular electrical measurements were done by one-electrode current- or voltage-clamp.

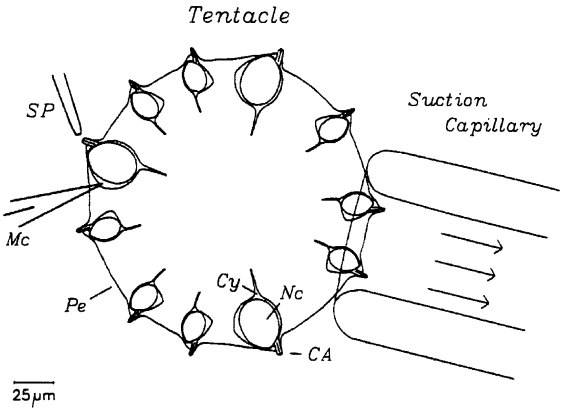

Figure 2. Scheme of the arrangement for stimulation and intracellular recording from nematocytes in isolated tentacular spheres of Corynidae (as seen in focal plane). CA, cnidocil apparatus; Mc, microcapillary electrode; Nc, nematocyst; Cy, cytoplasm; Pe, pellicle; SP, stimulating probe (from Brinkmann et al., 1996).

Results and discussion

Ultrastructure of the cnidocil apparatus

The regular concentric arrangement of the stereovilli surrounding a cilium differs from the eccentric configuration of vertebrate hair bundles (Fig. 1c). In vertebrates, tip links connect stereovilli of different length and are assumed to control the mechanosensitive ion channels (Hudspeth, 1989). They are not present in hydrozoan concentric hair bundles as our comparative studies with various methods showed. This is associated with the fact that the stereovilli are arranged in one ring only and are mostly of the same length. At their tips, they are tightly interconnected with their neighbors, thus forming a stiff cone (Fig. 1b). When force is applied to the cone from the side, it is not deflected with internal shearing but is tilted as a whole (Brinkmann et al., 1994). In its center, the cilium or cnidocil is radially linked to the stereovilli by chains of extra- and intracellular elements that connect the ciliary microtubular doublets to the microvillar actin core. These chains traverse the ciliary and stereovillar membranes (Golz & Thurm, 1991; Brinkmann et al., 1994; Golz, 1994; Thurm et al., 1998a).

The mechanosensory input of nematocytes

A purely mechanical stimulation of a nematocyte normally does not elicit the discharge of its cnidocyst. Therefore, the electrical and sensory properties of nematocytes could be studied with repetitive stimulation. Since only stenotele cells were studied intracellularly (they are the only type in Corynidae), the data shown may not be valid for all types of nematocytes.

Forces directed against the cnidocil apparatus cause phasic-tonic voltage responses, of depolarizing sign only (Brinkmann et al., 1996; Lawonn & Thurm, 1998; Fig. 3). The adaptation that occurs during about 200 ms of response decays within 200–500 ms after the end of the stimulus (shown by test stimuli). The peak response amplitude can reach 50 mV in *Stauridiosarsia*. The responses are due to an increase in nonspecific cation conductance with a reversal potential around 0 mV. They can be blocked by the ion-channel blocker streptomycin. These properties as well as the kinetics (latency <50 μs) are typical for mechanoreceptor potentials and similar to those of vertebrate hair cells (cf. Hudspeth, 1989). But different from vertebrate hair cells, nematocytes have no directional dependence of sensitivity. This corresponds to the radial symmetry of their cnidocil apparatus at the level of the radial links.

Action potentials are rare events in purely mechanically stimulated nematocytes. Only a single one can occur superimposed on a receptor potential of largest amplitude of Corynidae (see Brinkmann et al., 1996; Thurm et al., 1998b), but its occurrence is mostly restricted to combined mechanochemical stimulation and is correlated with the discharge of the cnidocyst (see below).

Two mechanical sensitivities were found in the cnidocil apparatus of *Stauridiosarsia* (Brinkmann, 1994; Brinkmann et al., 1994) as well as *Hydra* (Lawonn & Thurm, 1998; Lawonn, 1999): (1) sensitivity for deflection and longitudinal shift of the cnidocil versus the cone of stereovilli (Fig. 3a and b); and (2) sensitivity for force acting on the tips of the stereovilli ('touch') (Fig. 3c and d). A deflection (tilt) of the stereovillar cone by force applied at its side, which tilts the cnidocil concomitantly, is ineffective (see ultrastructure of the cone, above). The sensitivity of the stereovilli could be studied separately since the cnidocil, like most cilia, can be shed by the cell and regenerated within a few hours (Golz & Thurm, 1990). In *Hydra* the saturating response amplitude obtained from the stereovilli without cnidocil is only about

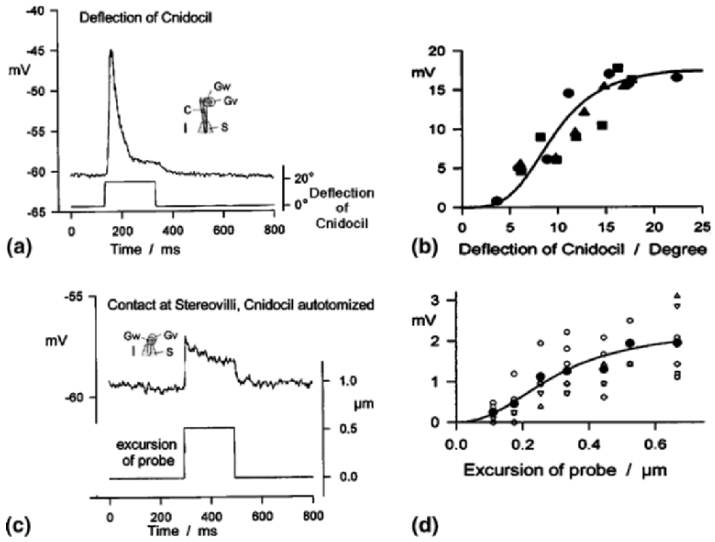

Figure 3. a and c. Receptor potential responses (upper traces) of stenotele nematocytes of *Hydra vulgaris* to mechanical stimuli shown in the lower trace. Inset-diagram a. Stimulatory force attacking at the cnidocil, c; Inset-diagram c. Force applied at the stereovillar tips, s, after loss of the cnidocil; G_v, G_w, probe in the resting and in the stimulatory position, respectively; scale bar: 1 μm. Diagrams b and d. Stimulus–response curves for the peak response amplitudes of records as in a(b) and c(d). Each diagram from three cells (recordings by T. Sieger).

10% (ca. 2 mV) of that obtained by deflecting the intact cnidocil (≤20 mV). Interestingly, there is only little adaptation in the responses of the stereovilli whereas the response evoked by selective deflection of the cnidocil adapts considerably (Fig. 3a and c). Nevertheless, the tonic responses for cnidocil deflections can be larger than those found in the stereovilli after loss of the cnidocil.

The different cell components and the different modes of deformation that underlie these different modes of response suggest that two different mechanisms of mechanoelectric transduction coexist in these concentric hair bundles (Thurm et al., 1998a). Monoclonal antibodies in *Hydra vulgaris* directed against the extracellular filamentous components of the radial chains connecting cnidocil and stereovilli strongly reduced mechanosensitivity of the nematocytes when and only when the antibodies were applied during regeneration of the links (Golz & Thurm, 1992; Lawonn et al., 1995). We conclude from the structural and physiological results that the radial filamentous links transmit the stimulatory force from the cnidociliary shaft to stress-sensitive ion channels. These channels are most likely components of the radial chains at their site of traversing the membrane of the cnidocil and possibly of the stereovilli (Thurm et al., 1998a; Fig. 1b). Besides this main component of sensitivity, a second mechanosensitive mechanism transduces local force acting on that stereovillar membrane area that covers the ends of the cytoskeletal actin filaments.

Synaptic signal transmission

Purely mechanical stimulation of the cilia of sensory hair cells and the cnidocils of nematocytes induces two different types of depolarizing current and voltage changes in distant unstimulated nematocytes of Corynidae (Brinkmann, 1994; Oliver & Thurm, 1996; Thurm et al., 1998b). Both types can reach amplitudes of 40–50 mV. *Type T* depolarizations are uniformly transient, more or less periodic and occur in loose correlation with various kinds of stimulation (Fig. 4; cf. Purcell & Anderson, 1995). *Type L* depolarizations occur correlated with the receptor potentials of stimulated nematocytes; they are graded in amplitude and duration, but their kinetics are considerably slower than those of receptor potentials. Most interestingly, if a nematocyte is stimulated repetitively, the latencies of L-depolarizations occurring in other cells change considerably: Without concomitant chemical stimulation (see below) the

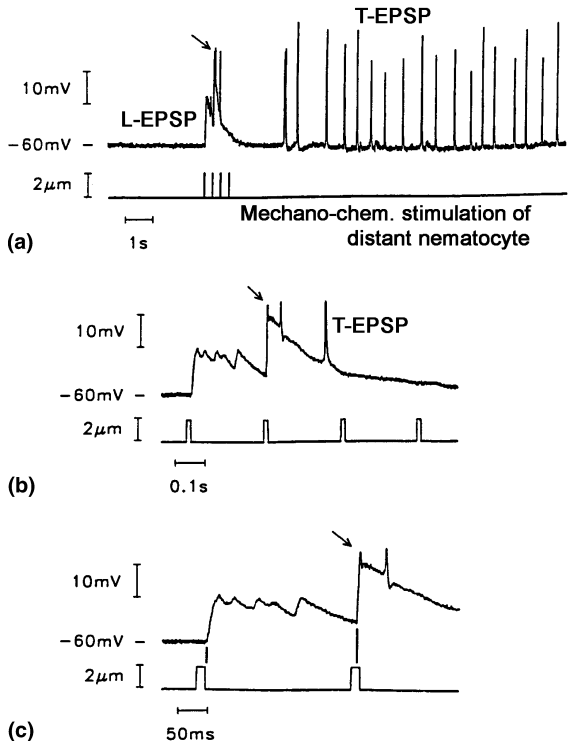

Figure 4. Depolarising postsynaptic responses (upper trace, L-EPSP and T-EPSP) in a nematocyte of *Stauridiosarsia producta* evoked by mechanochemical stimulation (lower trace, four rectangular stimuli) of a distant nematocyte in the same tentacle; probe coated with phosphatidylcholine. a, b, c, Segments of the same period of stimulation at increasing time resolutions (from a to c). The second stimulus evoked an increased L-EPSP; note the associated decrease of latency (see time resolution c). During the corresponding response peak, the stimulated nematocyte discharged its cnidocyst (arrows). This discharge is followed by a series of T-EPSPs (see a) (from Brinkmann, 1994).

latencies increase from about 10 ms in the first response up to 30 ms for later responses; with combined chemical stimulation they decrease down to 2 ms. The amplitudes of these responses change inversely to the latencies, while the stimulated cell is likely to produce uniform receptor potentials (Brinkmann, 1994; Oliver & Thurm, 1996).

The following observations shed light on the pathways that may contribute to these signal transmissions: *electrical coupling via gap junctions* (e.g. in an epithelial pathway) and/or *chemical synapses*:

- No gap junctions have been found at nematocytes (while they were found at the surrounding supporting cells) by serial sectioning and transmission electron microscopy (Holtmann & Thurm, 2001b).
- The gap-junction-permeating stain Lucifer Yellow injected into nematocytes does not leak out of the cells in their live state (Brinkmann et al., 1996; Lawonn, 1999).
- Octanol, which blocks gap junction conductance, does not block the L-type but likely the T-type responses (Brinkmann & Thurm, unpublished).
- The latencies and time courses of both types of responses cannot be derived from receptor potentials by passive conductance but have features of postsynaptic potentials.
- Substitution of Ca^{2+} by Mg^{2+}, which generally blocks synaptic transmitter release, blocks L- and T-type responses, but does not block receptor potentials (Brinkmann, 1994; Sieger & Thurm, 1997).
- The glutamate (kainate) receptor antagonist NS 102 (5 μM) blocks the L-type responses, and possibly also T-type responses, while cholinergic and aminergic agents are without effect (Sieger & Thurm, 1997).
- L-glutamate (superfusion with 50 mM) depolarizes nematocytes tonically and reversibly (Sieger & Thurm, 1997).

These results indicate: Both types of responses are excitatory postsynaptic potentials (L-EPSP and T-EPSP); both chains of signal transmission involve at least one chemical synapse. This synapse must be located at the nematocyte. This means, for nematocytes of Corynidae one must claim the existence not only of efferent synapses, which have been demonstrated for other Hydrozoa (e.g. Hufnagel & Kass-Simon, 1988; Westfall, 1996), but also of afferent synapses. The latter are likely glutamatergic and, according to the time course of the postsynaptic response, ionotropic (i.e. ion channels are receptor-integrated). Gap junctions are likely to be not enclosed in the signal transmission for L-EPSPs, but they might be engaged in T-EPSPs.

Modulations of exocytotic outputs

When adequate chemical stimulation is combined with mechanical stimulation, the probability and

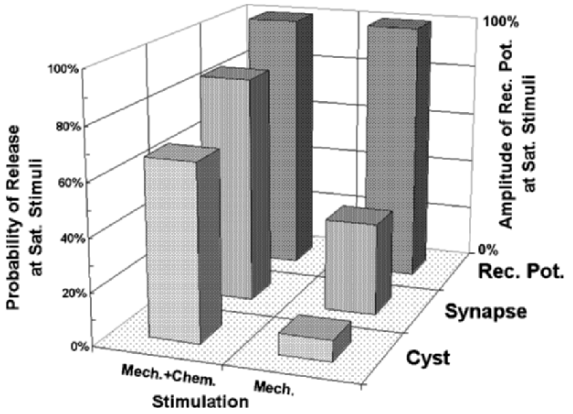

Figure 5. Influence of chemical sensitization on discharge of cnidocysts and delivery of synaptic signals (L-EPSP) at saturating mechanical stimuli in *Stauridiosarsia producta*. Rec. Pot., receptor potentials, normalized in relation to responses to purely mechanical stimuli. Probe coated with lecithin (mechanochemical stimulation) or without coat (purely mechanical stimulation). Number of cells stimulated mechanically 123; number of cells stimulated mechanochemically 249 (data from D. Oliver).

strength of afferent synaptic activity are drastically increased (Fig. 5). Simultaneously, the probability of discharge of the cnidocyst strongly increases (Brinkmann, 1994; Thurm et al., 1998b). Phospholipids are the only adequate natural stimuli to elicit these effects in stenoteles of Corynidae. For the more complex cnidom of *Hydra*, N-acetylated galactos- and glucosamine are most effective; water insoluble glycolipids, more than phospholipids, are efficient stimulants for the stenoteles (Lawonn & Thurm, 1992).

The water-insolubility of phospholipids (e.g. lecithin) allowed the localization of the site of their chemoreception: Only *direct contact* of the phospholipid-coated glass probe (diameter ca. 1 μm) with the cnidociliary shaft of a stenotele started the activation of this cell. Interestingly, these shafts are the only cellular structures that project out of the mucus or cuticular layer that otherwise covers all outer surfaces of the tentacles including the cones of stereovilli (e.g. Golz & Thurm, 1990 for *Hydra*; Holtmann & Thurm, 2001b for *Coryne*). A delay of about 1 s between the beginning of the contact and rise of discharge probability was found in *Hydra* (Thurm & Lawonn, 1990; Thurm et al., 1998b); a faster time course was found for synaptic signaling and discharge of the shorter stenoteles of Corynidae (Fig. 4; Brinkmann, 1994). We conclude that the sensitizing chemoreceptor molecules that get activated by water-insoluble glyco- and phosphatidyl-compounds are located in the membrane of the cnidocil of the studied hydrozoan stenoteles. This is in accordance with a high density of membrane particles in the cnidociliary membrane (Golz & Thurm, 1991). A high sensitizing efficiency of the water-*soluble* choline compound carbachol in Corynidae may reflect the properties of receptor elements that detect water-insoluble phosphatidyl choline (Thurm et al., 1998b). Chemoreception by the cnidocil, of course, does not exclude additional efferent effects of chemosensory cells activated by other substances and differing chemosensory effects on other types of nematocytes (Lentz & Barrnett, 1962; Lawonn & Thurm, 1992; Purcell & Anderson, 1995; Kass-Simon & Scappaticci, 2004).

The chemical stimulation itself does not cause a receptor potential within the stimulated nematocyte. But under contact-chemical stimulation the mechanoreceptor potential of larger cnidociliary deflections tends to develop a regenerating peak comparable to an action potential (Brinkmann, 1994; Lawonn, 1999). It likely triggers the simultaneous discharge of the cyst (cf. Gitter & Thurm, 1996).

Using the terminology developed for ganglion cells, the sensitizing effects of the contact chemical stimulation can be summarized as a chemo-sensory *modulation* that acts on the exocytotic mechanisms of the cnidocyst and the afferent synapses. Various experiments applying electrical stimulation at the nematocytes revealed that the modulation changes the electrical sensitivity of these membrane mechanisms such that the efficiency of their control by the receptor potential is increased (Thurm et al., 1998b).

Summarizing

The chemosensory modulation of the afferent signal release turns stenotele nematocytes into sensory detectors that respond to those coincidences of mechanical and chemical stimuli that are characteristic for the animal's prey (Thurm & Lawonn, 1990); in Corynidae, they additionally submit afferent signals that inform other cells about these mechanochemical detections and, by

an increased signal amplitude, about the nematocysts's discharge.

In *Hydra* (not in Corynidae), the state of being fed to repletion leads to a distinct *intrinsic* input to some of the modulatory mechanisms (cf. Smith et al., 1974). It strongly reduces the electrosensitivity of cyst discharge (Gitter & Thurm, 1993; Lawonn & Thurm, 1994; Thurm et al., 1998b). This effect can be mimicked by dopaminergic (D_2) agents.

A further intrinsic important control is exerted by the epithelial integration of nematocytes. The electrosensitivity of cyst discharge is absent when the cell is not integrated in the epithelium (cf. Anderson & McKay, 1987), while the cell's mechanosensitivity remains unchanged (Lawonn, 1999). This may be important for the fate of the cell during its subepithelial migration with an assembled cnidocil apparatus (Golz, 1995).

Synaptic ultrastructure

In Corynidae, the synaptic connectivity of sensory cells and of nematocytes is concentrated in the central volume of the tentacular spheres (Holtmann & Thurm, 2001b). The small size of the spheres of *Coryne tubulosa* (20–40 μm) allowed systematic study of the connectivity by electron microscopy of serial sections (Holtmann & Thurm, 2001a). Membrane contacts between neurites and nematocytes and sensory hair cells were found, with 3–10 clear and/or dense-core vesicles of 70–150 nm diameter attached to a membrane density at the side of the neurite (Fig. 6). These contacts correspond to the *efferent* synapses of nematocytes reported for other Hydrozoa (Westfall, 1996).

At the side of nematocytes and hair cells, clear vesicles were also attached to membrane densities in opposition to membrane contacts with a neurite or the soma of a hair cell or nematocyte, but these vesicles had diameters of 160–1100 nm ('magno-vesicles'). Their number per contact site was, inversely correlated with their size, 4–1 (Fig. 7). These diameters and numbers raise doubts about the synaptic function of these vesicles.

The following aspects are in favor of a synaptic function of these vesicles: Size and structure of the contact zones of the membranes are as common in cnidarian synapses; the total volume of all vesicles at a contact zone is in the usual range; the sizes and numbers of vesicles per contact overlap with those assumed to have synaptic function in coelenterates; the functional data require the existence of a rapidly working chemical synapse between various nematocytes, and hair cells and nematocytes; the described cell contacts are the only contacts which have some characteristics of

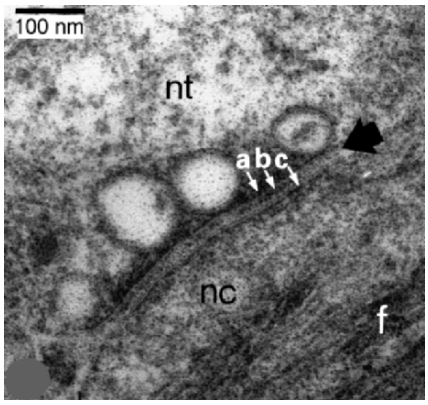

Figure 6. Electron micrograph of a putative neurite synapse (black arrow) that is *e*fferent to a nematocyte (nc) in a capitate tentacle of *Coryne tubulosa*. Four clear vesicles are cut on the neurite (nt) side. Layers of electron dense material on the plasma sides of each membrane and in the intercellular cleft are marked by a, b, and c. f, 10-nm filaments (from Holtmann & Thurm, 2001a).

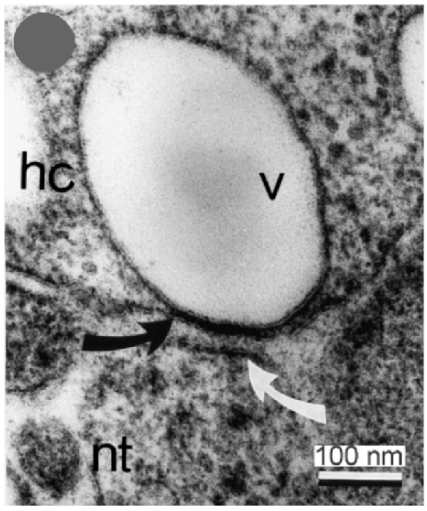

Figure 7. Electron micrograph of a putative neurite synapse that is *a*fferent to a mechanosensory hair cell (hc) in a capitate tentacle of *Coryne tubulosa*. One large clear vesicle is cut on the hair cell side (diameter ca. 400 nm 'magno-vesicle'). Nt, neurite; arrows indicate osmiophilic layers on the plasma sides of the opposed membranes (from Holtmann & Thurm, 2001a).

rapidly working chemical synapses not only in nematocytes but also in sensory hair cells, and they occur regularly at the same sites that are appropriate to explain the signal transmission found.

The discrepancy between the findings and the usual vision of a synapse is due to the principle 'one quantum of transmitter release per one vesicle.' This principle is incompatible with the consideration that a mono-(or oligo-)vesicular synapse might transmit signals repetitively and graded in amplitude and time (Thurm et al., 1999). Anderson & Grünert (1988) already pointed at this general problem for a cnidarian neurite–neurite synapse that has the usual small number of vesicles (oligo-vesicular synapse in contrast to the multivesicular synapses of vertebrates, for which the above cited principle had been worked out).

The discrepancy may be solved by recent results on the process of exocytosis of endocrine vesicles that have the size of magno-vesicles (Alvarez de Toledo et al., 1993): Instead of the usually assumed complete fusion of a vesicle, *transient* fusion and *partial* depletion of the transmitter was observed. Further results from mammalian central synapses indicate that smaller vesicles also can survive after release and be quickly refilled (Aravanis et al., 2003; Gandhi & Stevens, 2003). It remains to be explored whether these principles of *transient*, *reversible fusion*, *partial depletion*, and *continuous refilling* do occur at large *stationary* synaptic vesicles. (For implications of mono- and oligo-vesicular synapses, see Holtmann & Thurm, 2001a.)

The challenging indication of the extreme case of mono-vesicular synapses may reveal basic principles of cnidarian synapses in particular and steps in the evolution of synapses in general.

Acknowledgments

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Cnidarian neurobiology: what does the future hold?

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Abstract

Cnidarians have long been recognized as occupying a unique position in nervous system evolution and, consequently, have attracted considerable attention from neurobiologists over the years. During the latter half of the 20th century, the application of a variety of electrophysiological and other methods provided us with a great deal of information about the scope and composition of the cnidarian nervous system. Here, I will briefly review what is known about cnidarian nervous systems, what remains to be found and, most importantly, discuss the status and future of the field.

Introduction

As the earliest organisms to possess a recognizable nervous system, members of the Phylum Cnidaria, the sea anemones, corals, and jellyfish, attracted considerable attention over the years from neurobiologists and behavioralists. While recent molecular data (Podar et al., 2001) may no longer support their basal position in nervous system phylogeny, a position possibly occupied instead by ctenophores, cnidarians remain the group that provides the most tractable and useful preparations for studying the early nervous system. I have not used the term 'primitive nervous system' in this context, because the nervous system of any cnidarian has been evolving for as long as our own, albeit in a different manner. Nevertheless, because cnidarians have retained much of the structural simplicity of their early ancestors, they have always been viewed as a very useful window through which to examine the structure, composition, properties, and capabilities of one of the earliest nervous systems to have emerged.

This viewpoint raises the obvious question of what is to be gained by studying early nervous systems? Can modern representatives of the earliest nervous systems to emerge provide us with useful information about neurobiology in general, or, more to the point, have we learned anything useful in the 40 or so years since electrophysiological techniques were applied to these animals other than, perhaps, satisfying our curiosity about a very simple, very early group of animals? Equally important, today, what does the future hold for cnidarian neurobiology – more of the same or some significant contributions to neurobiology?

As this review will demonstrate, cnidarians have indeed yielded some generally useful information about fundamental neurobiological processes, but the amount they have generated is dwarfed by that from the more recognized model systems, most notably arthropods, nematodes, and molluscs. Furthermore, although cnidarians are simple organisms, with simple nervous systems and simple behavior, the enormous repertoire of techniques available for studying complex neuronal function greatly reduces the need for very simple models unless they provide additional strengths. Thus, some may view the cnidarian contributions of the past as being limited in scope, and, more importantly, regard the field as having little or no prospect of providing generally useful

neurobiological information in the future. As noted above, however, a major appeal of cnidarians has always been their evolutionary position, but for many, the evolutionary approach may, itself, be seen simply as curiosity-driven and unlikely to yield much in the way of useful information.

It is important to remember, however, that the most complex nervous system known, the human brain, has been molded by millions of years of nervous system evolution. While many factors will have contributed to the final product, and indeed it is arguable that higher central nervous systems may be polyphyletic, one of the most significant stages in that evolutionary history has to have been the form, composition, and properties of the earliest manifestation of a nervous system, something akin to that of a present-day cnidarian. This review will develop the thesis that the previous 40 years of research into the cnidarian nervous system (summarized in Fig. 1) is finally providing us with useful information about the full scope of that early nervous system, and about the cellular and physiological framework that evolutionary pressures molded into higher nervous systems. Most importantly, by applying the techniques of modern molecular and cell biology to that baseline data, we are now in a position to use that information to understand the factors and constraints that have helped mold the complex higher nervous systems and better understand aspects of the function of many of the components of those higher nervous systems.

Cnidarian neurobiology – the last 40 years

The earliest electrophysiological studies of cnidarians were extracellular recordings that took advantage of the fact that, in the case of hydroids at least, large electrical signals could be recorded very easily from a diversity of tissues. Studies by several investigators identified a variety of interacting conduction systems or pathways (for reviews, see Josephson, 1974; Stokes & Rushforth, 1979), and it became evident very quickly that the cnidarian nervous system has the capability of integrating information from a variety of conduction pathways. Activity in these conduction systems could often, but not always, be correlated to discrete behavioral activities.

The term ‘conduction system’ emerged largely because of uncertainty about the cellular substrate

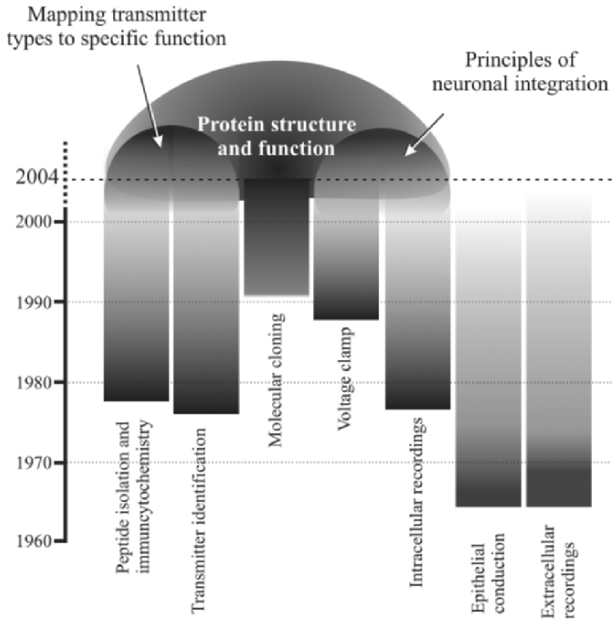

Figure 1. A time line depicting cnidarian neurobiological research activity over the past 40 years, with projections for possible future research foci, based on points discussed in this review. The level of interest/activity in a given area is indicated by the darkness of the bar.

underlying the recorded activity. At the time, the cnidarian nervous system was thought to be composed almost exclusively of diffuse nerve nets, and it was unclear how such large (up to 5 mV) electrical signals could be recorded so easily by suction electrodes applied to tissues that contained only a small number of fine neuronal processes. This uncertainty was compounded by the finding that epithelial cells of certain hydrozoans were electrogenic (Mackie, 1965, 1976; Mackie & Passano, 1968), and were capable of producing and transmitting electrical signals, a phenomenon termed 'epithelial conduction'. The discovery of epithelial conduction raised the possibility that the large events typically being recorded from hydroids could be either large nerve spikes, epithelial action potentials, or a combination of the two whereby underlying neuronal activity triggered an electrogenic event in the surrounding epithelia. This dilemma was perhaps best manifest in sea anemones where relatively large, slowly conducting impulses could be recorded from what were thought at the time to be nerve-free tissues. However, because the involvement of nerves could never be completely ruled out, a requirement for categorizing an epithelial conduction system, the pathways were given the rather amorphous titles Slow System 1 (SS1) and Slow System 2 (SS2) (McFarlane, 1969). This ambiguity about the cellular basis of electrical activity in cnidarians made the goal of being able to correlate these animals' simple behaviors with known networks of cells and pathways problematic. This inevitably led to a decline in activity in the field, particularly as other invertebrate models that permitted intracellular recordings from identified neurons were developed.

Two developments in the 1970s contributed to a re-emergence of interest in cnidarians: the discovery of a limited number of preparations that permitted intracellular recordings from identified neurons, and a greatly improved understanding of cnidarian neuroanatomy that emerged from immunocytochemical (ICC) studies directed at the many neuropeptides that were being discovered in these animals (for reviews, see Grimmelikhuijzen et al., 1988, 1989, 2002). The latter development changed our image of the cnidarian nervous system from one exclusively composed of diffuse nerve nets to more complex systems consisting of very obvious concentrations of neuronal elements in nerve rings and nerve tracts. That many of these were concentrated in the oral regions of the animal and were often associated with defined sensory structures raised the beguiling possibility that these animals may possess rudimentary brains or a CNS. This better understanding of the neuroanatomy of cnidarians also resolved uncertainty about the nature of the conduction pathways. In the case of sea anemones, for instance, it confirmed that the SS1 and SS2 systems were neuronal and not epithelial (McFarlane et al., 1987). Similarly, the presence of distinct nerve tracts where there had previously been thought only to be diffuse nerve nets helped explain the relatively enormous size of the electrical activity that had been recorded by extracellular electrodes.

Perhaps the biggest development, however, was the introduction of intracellular recordings, and later voltage clamp techniques, to cnidarian neurobiology. These advances had two important consequences. First, they demonstrated, for the first time, that cnidarian neurons and synapses operate in the same way as those in all higher animals: the neurons produce fast, overshooting action potentials that are now known to be produced by fast, inactivating Na^+ currents (Anderson, 1987; Spafford et al., 1996), and they are connected by fast chemical (Anderson, 1985) and, in the case of hydrozoans, also electrical (Spencer, 1981) synapses. Also, more recent work has shown that the oral nerve rings of *Aglantha* are the site of as much neuronal integration as the neuropile of any animal (Mackie & Meech, 1995a, b, 2000), belying the view that cnidarian nervous systems are composed of a limited number of distinct and diffuse neuronal pathways. The second benefit of the use of intracellular recordings was that it permitted cnidarian neurobiologists to begin to contribute to our understanding of general neurobiological principles. For example, the clear demonstration that structurally symmetrical chemical synapses in the scyphozoan jellyfish *Cyanea* are bidirectional chemical synapses (Anderson, 1985), as their structure implies, opened the door to understanding how structurally similar synapses in higher animals (Ewald & Roper, 1994) could function. Similarly, the discovery that giant axons in the hydromedusa *Aglantha* were capable of producing two types of

action potential (Mackie & Meech, 1985) provided support for a similar mechanism in vertebrate CNS neurons (Llinas & Jahnsen, 1982). Finally, the work of Spencer (1982) on the hydromedusa *Polyorchis* provided a very useful insight into mechanisms that could be used to synchronously activate large muscle fields.

It is important to note, however, that the application of intracellular recordings to cnidarians has been limited to a very few preparations. Indeed, in the 25 year since convincing intracellular recordings were obtained from cnidarian neurons, only four preparations have yielded useful information about cnidarian neurophysiology: the inner nerve ring of *Polyorchis* (Anderson & Mackie, 1977; Spencer, 1981), the giant fiber or motor nerve net of *Cyanea* (Anderson & Schwab, 1983; Anderson, 1985), various elements in *Aglantha* (Mackie & Meech, 1985, 1995a, b, 2000), and, to a lesser extent, the cubomedusa *Carybdea* (Satterlie & Spencer, 1979). All four preparations are characterized by the presence of unusually large (up to 40 μm diameter) neurons that can be visualized relatively easily. Other preparations undoubtedly exist in other medusae, but to date, no intracellular recordings have been obtained from neurons in polyps from any cnidarian classes, where neurons are consistently very small and difficult to identify in living tissue. This situation is particularly unfortunate with respect to *Hydra*; good physiological data would be a great complement to the wealth of developmental and cell biological information this genus has provided.

The use of voltage-clamp recordings from dissociated cells (Anderson, 1987; Przysiezniak & Spencer, 1992, 1994) coupled with molecular cloning have provided useful insight into the structure and function of cnidarian ion channels (Anderson et al., 1993; Spafford et al., 1996; Jeziorski et al., 1998), and the general picture that has emerged is that although there are functional differences, primarily from the perspective of pharmacology (Anderson, 1987; Spafford et al., 1996; Jeziorski et al., 1998), the structures of ion channels in cnidarians are remarkably similar to those that underlie the electrical activity of excitable cells in higher animals. This finding presents exciting possibilities for understanding ion channel structure and will be developed later in this review. To date, the pore forming subunits of voltage-gated Na^+ (Anderson et al., 1993; Spafford et al., 1996; White et al., 1998), K^+ (Jegla et al., 1995) and Ca^{2+} (Jeziorski et al., 1998) channels, together with one accessory subunit (Jeziorski et al., 1999) have been cloned from three classes of cnidaria. Where those channels have been expressed, their functional properties are consistent with those recorded *in vivo*.

What don't we know yet?

Despite the progress that has been made in our understanding of cnidarian neurobiology, several deficiencies or uncertainties remain, particularly in aspects of chemical and electrical synaptic transmission. In the case of electrical synapses, and most notably the issue of gap junctions, the situation has been perplexing. Gap junctions are very prevalent in hydrozoans. They were identified in the earliest electron micrographs of hydrozoan tissues and are known to form the basis of electrical synapses between neurons (Spencer, 1981), and in muscles and conducting epithelia (Josephson & Schwab, 1979). In contrast, however, clearly defined structural gap junctions have never been observed in anthozoans and scyphozoans, and until recently, there was no evidence for electrical or dye coupling between cells in these animals. This clear dichotomy between hydrozoans on the one hand, and anthozoans and scyphozoans on the other, has been the subject of much discussion in the field. Two hypotheses have been considered. First, functional gap junction proteins may indeed be present in scyphozoa and anthozoa but not in the assemblages that form readily recognizable gap junctions. Alternatively, anthozoans and scyphozoans may, indeed, lack gap junctions. In recent years, evidence has emerged to support the possibility that anthozoans, at least, possess gap junction proteins which, by definition, must be distributed too diffusely to generate classical gap junctions. The evidence in question comes from experiments with dissociated cells from *Renilla* (Germain & Anctil, 1996) and non-dissociated cells in tentacles from the anemone *Haliplanella* (Mire et al., 2000) that provided evidence for dye coupling between cells, and additional evidence that the coupling could be interrupted by agents, such as heptanol, which are known to uncouple

gap junctions. Finally, in the case of *Renilla*, it was shown that uncoupling agents also disrupt the physiological processes underlying bioluminescence.

Taken together, these findings suggest that intercellular coupling is, indeed, present in anthozoans and, by extension, suggest that it would likely also be present in scyphozoans, where appropriate studies have yet to be conducted. Even so, it is still intriguing why there is such a dichotomy in the extent to which the different classes use electrical coupling. Hydrozoans use epithelial conduction extensively, but no epithelial systems have been reported in anthozoans or scyphozoans, even in equivalent structures. For example, the exumbrellar surface of the bell of most hydromedusae is one large epithelial conduction system (Mackie & Passano, 1968; Josephson & Schwab, 1979) that provides mechanosensory information to the marginal nervous system. In scyphomedusae, the exumbrellar surface is innervated (Anderson et al., 1992). However, because that innervation is in the form of a diffuse, non-polarized nerve net, it provides the animal with no information about the specific site of stimulation on the exumbrellar surface, and in that sense, the quality of sensory information is no greater than that provided by an equivalent epithelial system. Nevertheless, the scyphomedusa must devote considerable additional resources (neurons) to achieve the same goal. Similarly, in hydromedusae, neurons in the nerve net that innervates the swimming muscle are tightly connected by gap junctions, and the entire nerve net functions as an electrical syncytium. In scyphozoans, however, the individual neurons that serve the same function are connected by fast, bidirectional chemical synapses that serve simply as fast interneural relays. Both architectures achieve the same result (for review, see Satterlie, 2002), a near synchronous activation of muscle over a broad area, but the situation in scyphozoans is surely more energetically costly to the animal since it involves multiple chemical synapses and a total of eight marginal ganglia. If gap junctional proteins were available to these animals, this would have been an ideal place to use them!

A particularly intriguing aspect of both studies of dye coupling in anthozoans (Germaine & Anctil, 1996; Mire et al., 2000) was the finding that antibodies to a non-conserved region of a rat cardiac gap junction protein (Connexin-43) stain appropriate molecular weight bands in Western blots of relevant tissues, and, in the case of *Renilla*, the same antibodies disrupt bioluminescence. These finding are interesting because connexins appear to be found exclusively in chordates; invertebrates appear to use only innexins (Phelan & Starich, 2001), which are structurally different. This may mean that connexins are more widespread than previously thought, or, more likely, may simply reflect the great care that must be taken in interpreting results obtained with phylogentically diverse antibodies.

The other area where our understanding of cnidarian neurobiology has not advanced as much as one might have hoped is in the area of neurotransmitters or, more correctly put, transmitters. This distinction is important because many substances that are used as neurotransmitters in higher animals may have had their origin as non-neural transmitter molecules and, until a given signaling substance is shown to be released from neurons, it cannot legitimately be called a neurotransmitter. Transmitters can be divided broadly into peptidergic and non-peptidergic classes. In the case of peptides, they are very prevalent in all cnidarian classes (for reviews, see Grimmelikhuijzen et al., 1988, 1989), some have been shown to have distinct physiological effects (McFarlane et al., 1987, 1991), and they have been localized in neurons, legitimizing their classification as neuropeptides. Unfortunately, progress in confirming the presence of non-peptidergic transmitters has been slow. Evidence for a variety of transmitters has been accumulated over the years. This includes evidence for cholinergic systems in a variety of species (Scemes, 1989; Falugi et al., 1994), serotonergic systems in the anthozoan *Renilla* (Umbriaco et al., 1990), dopaminergic systems in a sea anemone (Carlberg et al., 1984) and hydromedusa (Chung et al., 1989), glutamatergic transmission in anemones (Mendes & de Frietas, 1984) and *Hydra* (Kass-Simon & Scappaticci, 2004), taurinergic systems in the scyphomedusa *Cyanea* (Carlberg et al., 1995), nitrergic transmission in the hydromedusa *Aglantha* (Moroz et al., 2004), and, perhaps the most complete data set to date, monoaminergic transmission in the anthozoan *Renilla* (Anctil & Bouchard, 2004). In many cases, however, the

data are very limited, being restricted to behavioral observations, biochemical studies using whole animals, or immunocytochemical methods directed against components of neurotransmitter pathways using antibodies against mammalian components of those pathways. The result is that while there are tantalizing hints of transmitter pathways, in almost all cases the evidence is ambiguous.

Before a given transmitter candidate can be confirmed as a neurotransmitter, a rigorous set of criteria must be met (Eccles, 1957). This includes proof that the chemical in question is present in neurons, that it is released from those neurons under physiological conditions, that its application to the target tissue evokes the same response as the endogenous substance, and that physiological or pharmacological intervention to those applications mirrors the responses *in vivo*. In most cnidarian studies, only some of these criteria have been met, primarily because of technical difficulties presented by the small size and diffuse nature of the neurons. As a case in point, consider the identity of the neurotransmitter at synapses between MNN neurons in *Cyanea*. ICC (Carlberg et al., 1995) and HPLC studies (Anderson & Trapido-Rosenthal, 1990) have shown that the amino acid taurine is present in these neurons, and electrophysiological studies have demonstrated that applications of taurine to synapses produce depolarizing responses with the same reversal potential as the native EPSP (Anderson & Trapido-Rosenthal, 1990). Together these findings provide compelling evidence that taurine acts as a neurotransmitter at these synapses. However, convincing evidence that taurine is released following normal electrical activity is lacking, despite considerable effort (Anderson, unpublished). Although this preparation is one of the most suitable for the electrophysiological studies necessary to confirm the identity of neurotransmitters, final proof has been elusive. Thus, it should be no surprise that convincing evidence has been difficult to obtain in other species which provide far more difficult preparations. Another factor that complicates the search for neurotransmitters is transmitter pharmacology. As we have learned from pharmacological studies of cnidarian ion channels (Anderson, 1987; Spafford et al., 1996; Jeziorski et al., 1998), drugs that block or otherwise affect the action of a transmitter in a mammal may be completely ineffective against the same mechanism in a cnidarian. Thus, great care should be taken in interpreting pharmacological results, particularly negative ones.

One important consideration with respect to the variety of transmitters present in cnidarians is that there is no *a priori* reason why all the transmitter pathways present in higher animals should necessarily be used as neurotransmitters in cnidarians; some chemicals present in non-neuronal cells in cnidarians may have been recruited as neurotransmitters in higher animals. For example, judging by the ease with which many species can be stained for NADPH-diaphorase, the marker of nitric oxide synthase, nitric oxide pathways are probably quite common in cnidarians. However, with the exception of populations of neurons in *Aglantha* (Moroz et al., 2004), and the rhopalia of *Aurelia* (Moroz, personal communication), the staining is restricted to non-neuronal cells. Thus, neuronal NO pathways may be a relatively late development. Conversely, some neurotransmitter pathways present in cnidarians may have been lost in higher animals.

Despite the few uncertainties that remain, it is becoming increasingly clear that the cnidarian nervous system is composed of the same set of building blocks and components as higher nervous systems and functions in much the same way. Indeed, it is rather humbling to realize that our own nervous systems function in much the same way as that of jellyfish, with the only significant difference being the level of complexity.

The path ahead

Our understanding of the neurobiology of cnidarians has come a long way in the 40 years since modern electrophysiology and other techniques were first developed and applied. This quest has yielded a great deal of useful information about the scope and composition of one of the earliest extant nervous system, and the picture that has emerged is that the cnidarian nervous system is little different in most respects from higher nervous systems, just much simpler. Much of the effort that yielded this information was driven by simple scientific curiosity – a desire to learn how these very simple

animals are able to perform such a relatively elaborate repertoire of behavior given their very limited structure – coupled with the knowledge that cnidarians are among the simplest animals to possess a nervous system. However, it is arguable that much of the work to date has simply been the creation of a catalog of the parts of the cnidarian nervous system or, phrased more euphemistically, stamp collecting. While this type of inventory is critical to our understanding of the cnidarian nervous system, unless it is used well, it provides little information that is of interest to anyone but cnidarian neurobiologists or those broadly interested in nervous system phylogeny, and even then, only by providing an inventory of parts.

It is a reality of the times that curiosity-driven research is difficult to justify to both funding agencies and scientific peers, and, instead, it is now usually necessary to describe one's interest and justify a line of research in terms of the broad and generally important findings it will yield. Within this context, the question cnidarian neurobiologists must address is: does this group of animals offer opportunities for research that will yield generally useful and important neurobiological information, or will future research simply assemble a bigger catalog or stamp collection? If the latter, then it is very likely that the resources to support this research will dry up very quickly.

Having said that, however, I would argue that the field is now admirably positioned to make major advances in our understanding of basic neurobiological principles – advances that would not have been possible without the catalog that now exists. The vital consideration in this argument is still the important position that cnidarians occupy in nervous system, indeed, metazoan evolution. The cnidarian nervous system represents one of the earliest, if not the earliest nervous system known. At the same time, however, it may represent only one of several nervous system designs. Indeed, as noted earlier, the central nervous systems of higher animals may represent the assembly of many different nervous system designs, with the cnidarian nervous system likely representing the most basal component of that brain. This possibility, in itself, poses many opportunities for future research, but aside from this, there remain many opportunities for gleaning new and important neurobiological principles from these animals.

Some will come from looking at the cnidarian nervous system from a broad perspective. For instance, will a better understanding of (neuro) transmitter pathways in these animals reveal any fundamental correlation between transmitter type and function – serotonin and/or nitric oxide and feeding behavior, for example? Will further study of neuronal circuits underlying behavior in animals such as *Aglantha* yield useful underlying principles of neuronal integration?

Other important contributions will come from a more focused approach that is derived from the fact that all components of the cnidarian nervous system have been evolving independently of equivalent components in higher nervous systems, including our own, for something on the order of 700 million years (Morris, 1993). Furthermore, because we now know, from the catalog of cnidarian nervous system parts, that many of those components (e.g. ion channels, chemical synapses) function in much, if not exactly, the same way in cnidarians as they do in ourselves, they present a rare opportunity to better understand the relationship between the structure and function of proteins in both cnidarians and higher animals. As a case in point, consider what has been learned from a Ca^{2+} channel β subunit cloned from *Cyanea capillata* (Jeziorski et al., 1999). The β subunit of voltage-gated Ca^{2+} channels serves to modulate the activity of the pore-forming α_1 subunit in several ways (for review, see Walker & De Waard, 1998). Alignments of vertebrate β subunit amino acid sequences reveal that the proteins are highly conserved over very broad spans of the protein, making it exceedingly difficult to map discrete structures to known function. The β subunit from *Cyanea*, $CyCa_v\beta$ (Jeziorski et al., 1999), modulates an α_1 subunit from the same animal in much the same way as mammalian β subunits and, indeed, the two can be interchanged in heterologous expression studies with little functional difference. However, when $CyCa_v\beta$ is added to an alignment of mammalian β subunits that also includes a fly β subunit, which is likewise functionally conserved (Grabner et al., 1994), the levels of identity in the core region of the protein decrease by over 20% (Anderson & Greenberg, 2001). The important fact is that while the gene has been mutating, the function of all β subunits in the alignment is conserved. Thus, the conserved function must be

attributable to the residual conserved structure. Thus, by including a jellyfish and fly β subunit in the alignment we have reduced the 'noise' in the alignment by 20% and can now focus on what is left to discern clues about the structure and function of this protein. In essence, we are taking advantage of the enormous phylogenetic distance between cnidarians and mammals to improve the signal-to-noise ratio in our alignments. Another example of the utility of this approach comes from an examination of the β subunit binding site on the Ca^{2+} channel α_1 subunit, where sequence information in the jellyfish α_1 subunit (Jeziorski et al. 1998), in essence, provided the same conclusion as site directed mutagenesis (De Waard et al., 1996) of each of nine amino acids.

There is no reason why the same approach cannot be used with other proteins, including other ion channels, neurotransmitter receptors, components of second messenger pathways, and other signaling molecules where, based on either *in vivo* analysis, or heterologous expression, function is known to be conserved. The complementary approach can be used when function is known to change but structure is largely conserved by focusing on small areas of a protein, even single residues, in regions that are known to be involved in a particular function. In this case, the phylogenetic distance can highlight structural differences that could map to the functional differences, providing a means to map function to the structure of neurobiologically important proteins in higher animals and, thereby identify, for example, binding sites for drugs and other pharmacological agents that affect the function of a protein.

In both these approaches, a measure of cataloging will inevitably be required to assemble sequence and functional information about important proteins. However, it is imperative for the field that collection of the baseline data not be an end unto itself, but merely the first step in taking full advantage of what the phylogenetic gulf between cnidarians and mammals promises to provide for our overall understanding of neurobiology. Indeed, there is a very real possibility that unless cnidarian neurobiologists begin to think in very broad terms, plans for future research, be it at the molecular, physiological, or anatomical level, will not be realized for lack of interest by the scientific community and funding agencies. This would be a very unfortunate outcome given the enormous promise this group of animals still holds.

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Neural pathways and innervation of cnidocytes in tentacles of sea anemones

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Key words: Aiptasia pallida, synapses, nematocytes, spirocytes, neurotransmitters

Abstract

Our previously published studies are here reviewed detailing neuro-cnidocyte synapses, demonstrating putative neurotransmitter substances, and identifying complex neural pathways in sea anemones. Synapses were traced to their contacts on nematocytes and spirocytes by transmission electron microscopy of serial thin sections of tentacles. In five animals, cells containing microbasic *p*-mastigophores had synapses with clear vesicles, whereas cells containing basitrichous isorhizas had synapses with dense-cored vesicles, providing preliminary evidence for a selectivity of neurotransmitter types for different nematocysts. Either clear or dense-cored synaptic vesicles were also present at neuro-spirocyte contacts. Antho-RFamide immunoreactivity occurred in some anthozoan synaptic vesicles and immunogold labeling of serotonin was found at a neuro-spirocyte synapse. Neural pathways included direct innervation of spirocytes by sensory cells, sequential neuro-neuro-spirocyte and neuro-neuro-nematocyte synapses and reciprocal synapses involving axons of both sensory cells and ganglion cells. Such synaptic patterns resemble neuro-effector pathways found in higher animals and lay to rest the independent effector hypothesis for cnidocyte discharge in tentacles of sea anemones.

Introduction

Sensory cells and ganglion cells were described at the light microscopic level in sea anemones as early as 1879 by the Hertwig brothers. However, the innervation of nematocysts has been controversial for over a 100 years. Parker (1916) found that in tentacles and acontia of *Metridium* either mechanical or chemical stimuli would elicit nematocyst discharge, but only in the immediate region of the stimulus. Hence, he called nematocysts independent effectors that were not under neural control. Pantin (1942) observed that touching the tentacles of sea anemones with a glass rod coated with saliva produced abundant discharge of nematocysts, whereas either mechanical or chemical stimulus alone was not effective. Thus he stated that it took a combined mechanical and chemical stimulus of the apical ciliary cone of the nematocyte to cause the nematocyst to fire. This became the accepted independent effector hypothesis of nematocyst discharge. Later Watson and co-workers (Mire-Thibodeaux & Watson, 1994; Watson et al., 2000) found that it is the stereocilia of ciliary cones of sensory neurons that elongate in response to chemical stimuli and tune to frequencies of swimming prey before nematocysts are discharged. That suggested a role for the nervous system in nematocyst discharge, but the neural pathway involved was still unexplained. Recent work in my laboratory demonstrated ultrastructurally the presence of both neuro-nematocyte and neuro-spirocyte synapses in tentacles of sea anemones (Westfall et al., l998a, l999). The putative neural pathways and synaptic patterns associated with discharge of cnidocytes in *Aiptasia pallida* (Verrill, 1864) will be reviewed in this paper. Preliminary immunocytochemical evidence for the presence of Antho-RFamide and serotonin in synaptic vesicles of various sea anemones also will be discussed.

Neural pathways to nematocytes

Cross sections of tentacles of *Aiptasia pallida* reveal both large mastigophores and smaller basitrichs with ciliary cones containing a cilium surrounded by stereocilia. Sensory cells have similar ciliary cones (Westfall et al., 1998b). The inner ring of stereocilia arises from the sensory cells whereas additional rings have thinner stereocilia that come from the surrounding supporting cells. Longitudinal thin sections of microbasic *p*-mastigophores and basitrichous isorhizas (Mariscal, 1974) in sea anemone tentacles were traced by electron microscopy until neuro-nematocyte synapses were found. The synapses were characterized by the presence of clear or dense-cored vesicles associated with paired synaptic membranes traversed by a series of extracellular thin filaments. Intracellular thin filaments connected the bottom row of vesicles to the presynaptic membrane. The vesicles ranged from 62 to 122 nm in diameter and the cleft was 20–30 nm wide (Westfall et al., l998a).

The basoepithelial nerve plexus of the sea anemone tentacle is very complex. Therefore, tracing small unmyelinated nerve processes to their synaptic contacts on identifiable cells is extremely difficult. Our procedure was to cut 200–250 serial thin sections per tentacle and scan them in the electron microscope until synapses were located. We soon found that following the cnidocyte membrane near the basally located nucleus gave us the best results. Using this method, we observed nine neuro-nematocyte synapses in the tentacles of five animals. Of these, only clear vesicles were found at mastigophore-containing nematocytes, whereas dense-cored vesicles were present at basitrich-containing nematocytes. Although more data are needed, our preliminary anatomical results suggest that different types of neurotransmitters may be associated with different types of nematocysts. Furthermore, sequential neuro-neuro-nematocyte synapses were observed, providing anatomical evidence for presynaptic inhibition in the event that supporting physiological evidence may be found. In addition, reciprocal synapses were present between the two axons, suggesting that the neuro-nematocyte pathway may be modulated as occurs in the nervous system of higher organisms. Although we did not find identified ganglion cell innervation of the nematocytes in *Aiptasia*, we did see this in *Hydra* nematocytes (Westfall et al., 1971; Yu et al., l985). Also, in our limited study we did not establish a direct sensory cell-nematocyte contact as observed in *Hydra* (Westfall & Kinnamon, 1978). However, we found a direct contact of a sensory cell on a muscle cell (Westfall et al., 2002) which lends support to Parker's (1919) hypothesis of a two-cell neural pathway in the early development of the nervous system. Also, we found synaptic contacts on cnidoblasts, suggesting a trophic role for the nervous system in cnidocyte differentiation (Westfall et al., l998a). Future research using newer techniques and faster analytical methods will help elucidate the neural pathways controlling nematocyst discharge.

Neural pathways to spirocytes

The control of spirocyst discharge has long been an enigma to biologists. Spirocytes have no cilium or trigger hair, but these organelles occur in great abundance in sea anemone tentacles. Their capsules are quite thin with a finely serrated inner wall. The invaginated tubule is filled with hexagonal arrays of dense rods which upon eversion of the tubule form an array of fine adhesive microfilaments along one side of the tubule (Mariscal et al., 1977).

By following the basal region of the spirocyst-containing cells in the region of the nerve plexus we found 15 synapses in tentacles from five animals (Westfall et al., 1999). Ten of the neuro-spirocyte synapses contained clear vesicles ranging from 69 to 84 nm in diameter and five had dense-cored vesicles with diameters of 97–120 nm. In one example, both clear vesicle and dense-cored vesicle synapses were present on the same cell, suggesting that different types of transmitter substances may influence discharge of a single spirocyst. Furthermore, a sequential synapse was observed with dense-cored vesicles at the axo-axonal synapse and clear vesicles at the neuro-spirocyte synapse. Such variation in pattern of innervation may allow the modulation of spirocyst discharge. Our most interesting observation was of a sensory cell axon innervating both a spirocyte and a ganglion cell axon. The synapses were opposite and each contained dense-cored vesicles. Such synapses suggested both a direct two-cell pathway and an

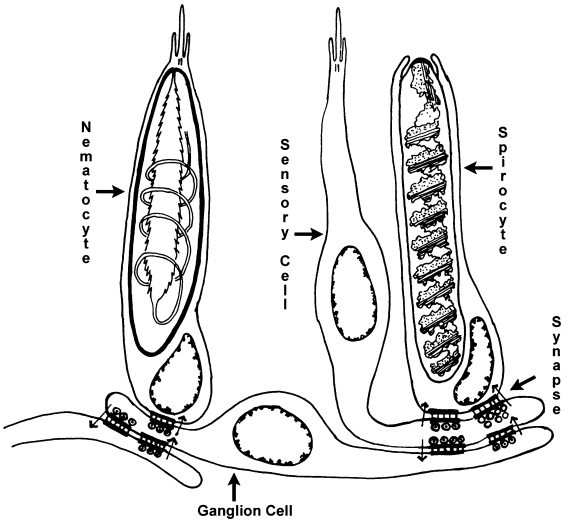

Figure 1. Schematic of two-cell (sensory cell-spirocyte) and three-cell (sensory cell-ganglion cell-nematocyte) neuro-cnidocyte pathways leading to capsule discharge in tentacles of sea anemones. (Surrounding supportive cells which contribute stereocilia to ciliary cones have been omitted for the sake of clarity). Synaptic pathways include direct neuro-effector cell contacts, sequential neuro-neuro-effector cell contacts, and reciprocal contacts between axons (see text).

indirect three-cell pathway involving one or more ganglion cells (Fig. 1). Excitatory and inhibitory responses may be controlled by sequential neuro-neuro-spirocyte synapses, whereas feedback between nerve cells may be regulated by reciprocal axo-axonal synapses. Because so many spirocysts are present in tentacles of sea anemones, it is probable that a single ganglion cell may innervate several spirocytes. Also, there are fewer sensory cells than mastigophores, suggesting that each sensory cell may innervate several nematocytes as we showed previously in *Hydra* (Yu et al., 1985). The tools are at hand for future researchers to elucidate more precisely the neural pathways involved in spirocyte discharge in sea anemones.

Transmitter substances in synaptic vesicles

Usually the vesicles associated with cnidocyte synapses could be classified as either clear or dense-cored. That suggests the presence of different types of neurotransmitters. The dense-cored vesicles indicate the presence of a peptidergic transmitter substance. We have localized Antho-RFamide immunoreactivity in some synaptic vesicles in *Anthopleura elegantissima* and Antho-RWamides I and II at neuromuscular synapses in *Calliactis parasitica* (Westfall & Grimmelikhuijzen, 1993; Westfall et al., 1995). Anderson et al. (1992) demonstrated Antho-RFamide immunofluorescence in neurons associated with a cluster of nematocysts in the jellyfish *Cyanea lamarkii*. We observed ganglion cells innervating clusters of nematocysts in the freshwater hydra (Yu et al., 1985) and found RFamide-like immunoreactivity over the granular cores of vesicles in epidermal ganglion cells (Koizumi et al., 1989). Thus one or more neuropeptides may be involved in the neuro-nematocyte pathway. Also, we localized anti-5HT antibody or serotonin at neuro-spirocyte synapses using both the peroxidase–anti-peroxidase (PAP) method and immunogold labeling of serial thin sections (Westfall et al., 2000). That suggests that the classical transmitter serotonin may be part of the neural pathway for cnidocyte discharge. Other possible transmitter substances found in anthozoans are dopamine and norepinephrine (Carlyle, 1969; Lenicque et al., 1977; DeWaele et al., 1987; Pani et al., 1995) but they have not been demonstrated to be associated with the neural pathway for cnidocyte discharge.

To date, we have no data to indicate the type of transmitter substance present in the clear or electron-lucent vesicles. So far, the presence of acetylcholine has not been established in sea anemone synapses. The classical neurotransmitters GABA and glutamate appear to play a role in nematocyst discharge in *Hydra* (Kass-Simon & Scappaticci, 2004). However, further studies are needed to identify the types of transmitter substance present at clear vesicle synapses associated with cnidocytes in sea anemones.

Summary and conclusions

Nerve cells affecting cnidocyte discharge in sea anemones consist of sensory cells that respond to vibration patterns of swimming prey and ganglion cells that participate in through-conducting pathways that may inhibit or modulate cnidocyte responses. In a local reflex arc, a sensory cell might trigger a spirocyte to fire its spirocyst in response to appropriate vibratory and chemical stimuli from its prey. However, to capture prey a three-cell

pathway involving a ganglion cell as an interneuron with integrative and motor functions is likely involved. The synaptic patterns include sequential synapses, which may indicate the presence of presynaptic inhibition by the sensory cell or ganglion cell. Ganglion cells may also inhibit sensory cells from triggering cnidocyte discharge in well fed sea anemones. Neuromodulation is possible via adjacent reciprocal axo-axonal synapses associated with the neural pathway to cnidocyte discharge.

The innervation of the different types of cnidocytes follows a similar pattern in nematocytes and spirocytes even though the cells are distinctly different. Ciliary cones are present on nematocytes which fire a paralyzing poison into the prey, whereas no ciliary cone is present on spirocytes which release a sticky thread to hold the prey. Yet the synapses to each type of cnidocyte contain a pattern of clear or dense-cored vesicles having similar diameters. Both types of cnidocytes have sequential and reciprocal synapses which provide anatomical evidence for the possibility of presynaptic inhibition and interneuronal feedback or neuromodulation.

In conclusion, electron microscopy of cnidocyte synapses in tentacles of sea anemones has provided preliminary evidence for two-cell and three-cell neural pathways with modulatory patterns and variation in transmitter substances. This allows us to put to rest the independent effector hypothesis for nematocyst discharge and to include neural control of spirocyst discharge in sea anemones.

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Dynamic tuning of hair bundle mechanoreceptors in a sea anemone during predation

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Key words: nematocyst discharge, mechanotransduction, hair cell, chemoreceptor

Abstract

The sea anemone *Haliplanella luciae* (Cnidaria, Anthozoa) detects chemical and mechanical stimuli from prey. Hair bundle mechanoreceptors on the tentacles participate in regulating discharge of microbasic p-mastigophore nematocysts. Properly stimulated hair bundles sensitize the anemone to discharge nematocysts into objects that contact the tentacles. The hair bundle mechanoreceptors are composed of stereocilia derived from a multicellular complex. This complex consists of a single sensory neuron surrounded by two to four supporting cells. The mechanoreceptor is similar in many ways to vertebrate hair cells of the acousticolateralis system. However, anemone hair bundles are adjustable in structure and responsiveness according to the activity of two different chemoreceptors. One chemoreceptor binds *N*-acetylated sugars and the other binds amino compounds including proline. *N*-acetylated sugars induce lengthening of the hair bundle and a downward shift in frequencies that elicit maximal discharge of microbasic p-mastigophore nematocysts. Furthermore, *N*-acetylated sugars shift maximal discharge to smaller amplitude vibrations. Thus, *N*-acetylated sugars likely tune hair bundles so that small, swimming zooplankton stimulate maximal discharge. Proline leaks into the seawater from the hemolymph of wounded prey. Proline induces shortening of the hair bundle and shifts maximal discharge of nematocysts to higher frequencies and to larger amplitude vibrations. Thus, proline likely tunes hair bundles so that small, wounded, prey stimulate maximal discharge of nematocysts as they struggle to escape. Thus, suitably sized prey stimulate maximal discharge of microbasic p-mastigophore nematocysts upon first contacting the anemone tentacle and again upon attempting to escape.

Introduction

Predator/prey relationships are among the most fascinating relationships among animals. The sea anemone *Haliplanella luciae* is a successful predator of zooplankton. Upon detecting suitable prey, nematocytes become sensitized to discharge nematocysts in response to contact between the prey and anemone. Prey are detected by means of chemical and mechanical stimuli. The first chemical stimulus, *N*-acetylated sugars including N-acetylneuraminic acid (=NANA), commonly occurs in glycoproteins. Glycoproteins having *N*-acetylated sugars occur as integral proteins of the cell surface or in extracellular proteins such as those occurring in mucous coverings. Because *N*-acetylated sugars are so common, they may signify merely the close proximity of an object that is living, was once living, or produced and secreted by a living thing. *N*-acetylated sugars located in mucous coatings diffuse from the prey and bind to chemoreceptors located on the tentacles of the anemone. Chemoreceptors for *N*-acetylated sugars occur at the apical plasma membrane of epithelial cells called supporting cells (Watson & Hessinger, 1987, 1988, 1989a). Supporting cells are the

dominant cell type of the tentacle epithelium. Supporting cells always surround cnidocytes. Thus, they are at least two to four times more abundant than cnidocytes. In this context, it is clear that anemones dedicate considerable resources to chemosensing prey. However, supporting cells are not limited to a single function. Supporting cells also are involved in detecting vibrations. Vibrations produced by swimming prey constitute the first mechanical stimulus that can be detected by the anemone. Vibrations are detected by hair bundle mechanoreceptors. Anemone hair bundles, like those of the acousticolateralis system, are composed of stereocilia (Watson & Roberts, 1995). Stereocilia are finger-like projections of the plasma membrane that overlie an actin-based cytoskeleton (Tilney et al., 1980; Hirokawa & Tilney, 1982). Like vertebrate hair bundles, anemone hair bundles have tip links interconnecting the tip of one stereocilium with the distal side of the adjacent, taller stereocilium (Pickles et al., 1984; Watson et al., 1997). According to the currently favored model for signal transduction in vertebrate hair cells, tip links are likely to be gating springs that directly attach to transduction channels at one or both ends of the tip links (Howard et al., 1988). Deflecting the hair bundle in a direction that imposes strain on the tip link opens the channel. Deflecting the hair bundle in a direction that allows slack on the tip link closes the channel.

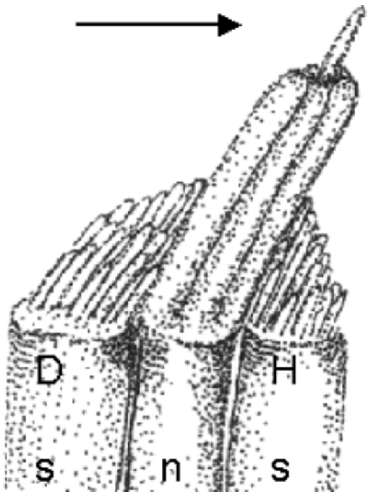

Figure 1. Idealized diagram depicting an anemone hair bundle derived from a single, central, sensory neuron (n) and four supporting cells (s), of which two are shown. A deflection of the hair bundle in the direction of the arrow causes stereocilia on the supporting cell to the left to be deflected toward the sensory neuron. Consequently, strain on that supporting cell's gating springs opens its transduction channels, allowing a cation influx leading to depolarization (D) of the membrane potential. The stereocilia on the supporting cell on the right side of the sensory neuron are deflected away from the sensory neuron. Slack on its gating springs closes the transduction channels, preventing a cation influx and leading to hyperpolarization (H) of the membrane potential.

Hair bundle mechanoreceptors in anemones

Several points of similarity between anemone and vertebrate hair bundle mechanoreceptors have been described in detail elsewhere (Watson et al., 1997; Watson & Mire, 1999). One of the key differences, however, between anemone hair bundles and those of the acousticolateralis system is that anemone hair bundles are composed of stereocilia derived from a multicellular complex (Mire-Thibodeaux & Watson, 1994). A single sensory neuron at the center of the complex contributes six large diameter stereocilia and a kinocilium to the hair bundle. The surrounding supporting cells contribute several hundred small diameter stereocilia to the hair bundle (Fig. 1). Because the supporting cells have the majority of stereocilia, they are responsible for conducting the majority of the transduction current (Mire & Watson, 1997). According to the working model, anemone hair bundles are omnidirectionally sensitive. A target vibrating at a suitable frequency causes the hair bundle to oscillate about its base. Considering one half of this oscillatory movement, the hair bundle pivots from a resting position to a bent position. The supporting cells on either side of the neuron experience very different changes in their orientation (Fig. 1). One supporting cell has stereocilia that bend towards the sensory neuron. This movement produces strain on the tip links causing the channels to open and allowing a cation influx. The cation influx promotes depolarization of the membrane potential in the supporting cell. The supporting cell on the opposite side of the sensory neuron has stereocilia that bend away from the sensory neuron. This movement allows slack on the tip links causing the channels to close and preventing a cation influx. The membrane potential of this supporting cell hyperpolarizes.

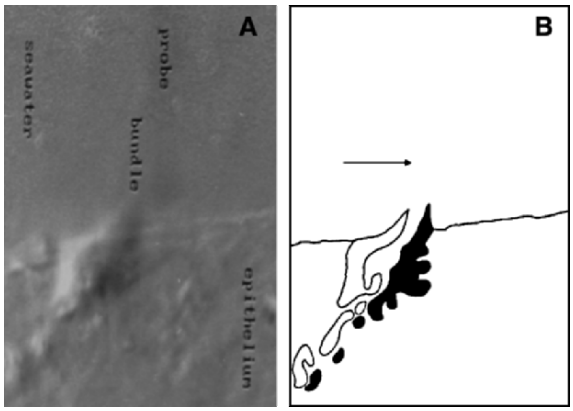

Figure 2. Ratio image constructed from digitized epifluorescence micrographs of a living, excised tentacle loaded with DI-8-ANEPPS, a fluorescent dye sensitive to membrane potential. The two images, A and B, were taken approximately 2 s apart, immediately before and after a step deflection of an anemone hair bundle. (A) No significant change in fluorescence intensity is represented as grey. Significant brightening (membrane depolarization) appears white and significant dimming (membrane hyperpolarization) appears black. The position of the hair bundle and probe are indicated on the micrograph. (B) Tracing of the micrograph to clearly define the brightening (white) in one supporting cell and dimming (black) in the supporting cell located on the opposite side of the same hair bundle. The arrow indicates the direction in which the hair bundle was deflected.

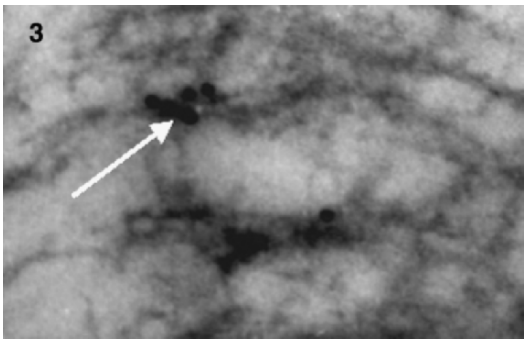

Figure 3. Post-embedment electron immunocytochemistry micrograph of the tentacle epidermis. Sections were stained using a monoclonal antibody for connexin 43 followed by antimouse IgG conjugated to colloidal gold. Immunopostive membranes (arrow) occur in the basolateral domains of epidermal cells.

This model is supported by experiments in which specimens were loaded with the potential-sensitive, fluorescent dye DI-8-ANEPPS (Molecular Probes, Eugene, OR). The dye fluoresces more intensely upon depolarizing the membrane potential and less intensely upon hyperpolarizing the membrane potential. A step deflection of the hair bundle causes the fluorescence to brighten on one side of the hair bundle and to dim on the opposite side of the hair bundle. This is shown in a ratio image in which the after-deflection image was divided by the before-deflection image (Fig. 2A). The two images were separated in time by only a few seconds. The ratio image is not crisp because the original fluorescent images of the specimen were obtained using an intensified video camera. Nevertheless, the distinction between the epithelium and seawater is obvious (Fig. 2A). The position of the probe that deflected the hair bundle and the apex of the hair bundle cannot be discerned and, consequently, are indicated by text on the micrograph. The micrograph should be analyzed as follows: A ratio of 1 (depicted as grey in the micrograph) indicates no change in fluorescence intensity between the before- and after-deflection images. Whereas most of the image is grey, indicating no change or little change in membrane potential, the hair bundle and the underlying cytoplasm of the supporting cells show brightening on one side of the hair bundle (corresponding to a ratio > 1) and dimming on the opposite side of the hair bundle (corresponding to a ratio < 1). In Figure 2B, the largest areas of brightening and dimming traced from Figure 2A are shown as white and black patches, respectively. In addition, an arrow indicates the direction by which the hair bundle was deflected (Fig. 2B). The ratio image indicates that a single, step deflection of the hair bundle produces oppositely directed changes in membrane potential of the supporting cells located on opposite sides of the hair bundle.

The depolarizing supporting cell communicates with the sensory neuron and other epithelial cells through dispersed (i.e. not clustered into plaques) gap-junction-like channels (Mire et al., 2000). These channels are immunopositive for antibodies raised to connexin 43 (Fig. 3) and inhibited by heptanol. Similar junctions were first reported in the sea pansy, *Renilla* (Germain & Anctil, 1996).

Regulation of discharge of nematocysts

Non-vibrating targets

The microbasic p-mastigophore is the most abundant type of nematocyte in the epithelium of feeding tentacles (Watson & Mariscal, 1983). Sea anemones discharge relatively few microbasic p-mastigophore nematocysts into non-vibrating, inanimate targets. For convenience, this response will be referred to as 'baseline discharge'. *N*-acetylated sugars sensitize discharge of nematocysts into non-vibrating test probes in a dose-dependent fashion (Watson & Hessinger, 1992). At the optimum concentration of *N*-acetylated sugar, the number of discharged nematocysts typically doubles in relation to baseline to the maximum matching the density of microbasic p-mastigophore cnidocytes in the tentacle epithelium. Thus, anemones readily discharge nematocysts into non-moving and even non-living prey based solely on the chemical stimulus.

Vibrating targets – frequency tuning

A mechanical mimic of a suitable, swimming, prey organism (i.e. a test probe vibrating at a frequency normally produced by a calmly swimming prey) is insufficient to sensitize nematocyst discharge above baseline. Thus, an appropriate mechanical stimulus alone is insufficient to sensitize discharge. Strangely enough, vibrations produced at unnaturally high frequencies (i.e. at frequencies higher than produced by an appropriate prey) stimulate maximal discharge of nematocysts (Watson et al., 1998). Thus, vibration sensitivity is present in the absence of *N*-acetylated sugars, just frequency-tuned to higher than natural frequencies. In the absence of *N*-acetylated sugars, maximal discharge occurs at 51, 55, 65–67, and 74–75 Hz (tested over the range of 0–75 Hz). In the presence of 10^{-7} M NANA, maximal discharge shifts to 1–7, 10–11, 14, 24–37, and 42–43 Hz (Watson et al., 1998). These frequencies overlap those produced by larval crustaceans including *Artemia* (Watson & Hessinger, 1989b). Thus, *N*-acetylated sugars shift maximal discharge of nematocysts to lower frequencies matching those produced by appropriate swimming prey. Vibrations at frequencies other than optimal frequencies yield baseline levels of discharge despite the presence of NANA (Watson et al., 1998).

Vibrating targets – amplitude tuning

In the absence of *N*-acetylated sugars, maximal discharge of nematocysts occurs over a broad range of amplitudes (Watson & Hudson, 1994). For targets vibrating at 55 Hz, maximal discharge occurs at ± 50–150 μm. The $\pm$ signs indicate movement of the probe from a resting position in a positive direction, returning to rest, and then moving in a negative direction. For example, an amplitude of ± 50 μm describes probe travel 50 μm in a positive direction, followed by 50 μm in a negative direction. Probe movements were calibrated in water to a depth corresponding as closely as possible to testing conditions. For targets vibrating at 65 Hz, maximal discharge occurs at ± 50–325 μm. At 75 Hz, maximal discharge occurs over the entire range tested from ± 50 to 350 μm. In the presence of NANA, maximal discharge shifts to lower frequencies. At 5 Hz, maximal discharge occurs at ± 75 μm (Watson & Hudson, 1994). This finding is significant in that the largest amplitude movements of a swimming organism occur at the lowest frequencies. Thus, *N*-acetylated sugars sharply narrow amplitude specificity to relatively small amplitude vibrations. In essence, *N*-acetylated sugars tune maximal discharge to movements matching those produced by relatively small zooplankton.

We wondered how *N*-acetylated sugars affected vibration sensitivity. By examining live specimens using video enhanced light microscopy, we found that NANA induced lengthening of hair bundles originating from sensory neurons and their surrounding supporting cells and induced no detectable change in length in ciliary cones (originating from cnidocytes and their surrounding supporting cells) (Mire-Thibodeaux & Watson, 1994). Furthermore, in the combined presence of NANA and specific, low concentrations of cytochalasin D that block polymerization of G-actin into F-actin, no significant elongation of hair bundles was observed. Likewise, vibration dependent discharge of nematocysts did not shift to lower frequencies in the combined presence of NANA and cytochalasin D (Mire-Thibodeaux & Watson, 1994). Thus, the downward shift in frequency responsiveness nor-

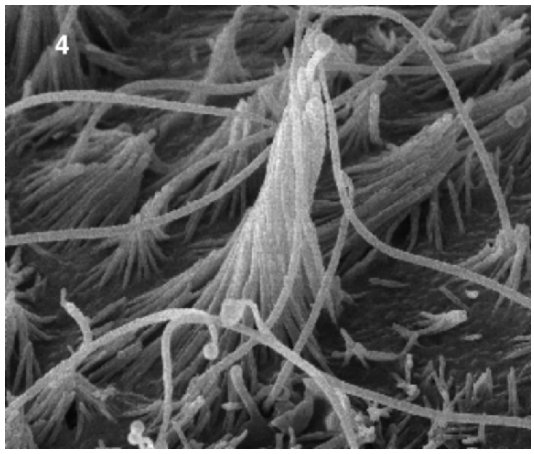

Figure 4. Field emission scanning electron micrograph of an anemone hair bundle from a control animal. Small diameter stereocilia wrap around the long axis of the hair bundle.

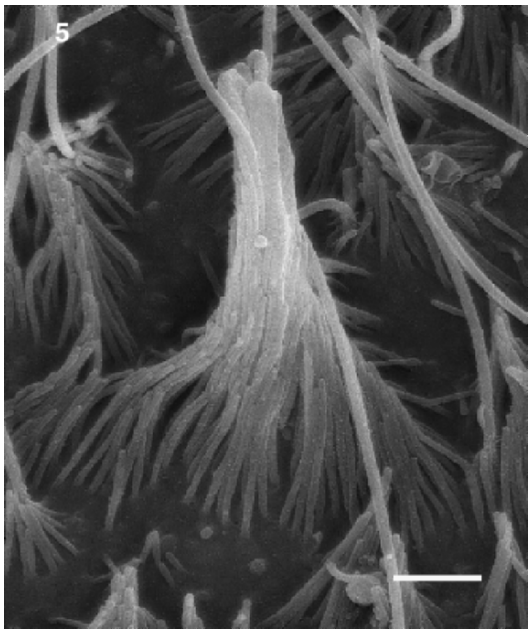

Figure 5. Field emission scanning electron micrograph of an anemone hair bundle from an animal exposed to NANA prior to fixation. Small diameter stereocilia tend to lie onto the large diameter stereocilia without wrapping around large diameter stereocilia from the sensory neuron. Scale bar = 2 μm.

mally induced by NANA requires an elongation of the hair bundle, apparently involving a net increase in F-actin. Reciprocal changes in levels of G-actin (decreasing) and F-actin (increasing) induced by NANA were confirmed in hair bundles by confocal microscopy (Watson & Roberts, 1995). An analysis of electron micrographs prepared from specimens fixed before and after NANA exposure indicated a likely increase in F-actin in large diameter but not small diameter stereocilia induced by NANA (Watson & Roberts, 1995). More recent investigations indicate that NANA induces a change in the arrangement of small diameter stereocilia. In untreated controls, small diameter stereocilia are markedly twisted around the large diameter stereocilia. The single kinocilium surrounded by the six large diameter stereocilia define the long axis of the hair bundle. Upon NANA stimulation, the small diameter stereocilia straighten (Mire & Nasse, 2002; Figs 4 and 5). The straightening of small diameter stereocilia is blocked by low levels of cytochalasin D. Hence, straightening is dependent on actin polymerization. NANA exposure increases mechanoelectric responses of hair cells to deflection of their hair bundles (Mire & Nasse, 2002). We believe that this shift in responsiveness (i.e. this increase in membrane current) is linked to the shift in amplitude sensitivity that accompanies exposure to NANA such that maximal discharge occurs into probes vibrating at relatively small amplitudes.

Amino compounds including proline constitute the second chemical stimulus. Receptors for proline co-occur with those for *N*-acetylated sugars at the plasma membrane of supporting cells (Watson & Roberts, 1994). The effects of proline on vibration-dependent discharge of nematocysts require prior exposure of the animal to *N*-acetylated sugars (Watson & Hessinger, 1994). Proline induces shortening of hair bundles and a loss of F-actin content (Watson & Roberts, 1995). Proline shifts maximal discharge of nematocysts to higher frequencies (Watson & Hudson, 1994). In addition, proline shifts maximal discharge to larger amplitudes. Thus, maximal discharge at 5 Hz occurs at ± 75 μm in NANA alone. In animals stimulated by NANA followed by NANA and proline, maximal discharge at 5 Hz shifts to ± 200 μm. Since proline occurs in the hemolymph of the prey, local increases in proline levels would follow wounding of the prey by penetrant nematocysts.

Discussion and conclusions

Potential sources of nutrition (alive or dead) are identified by the presence of *N*-acetylated sugars. If vibrations accompany the *N*-acetylated sugars, then it is clear that the potential prey is living. Only in the presence of *N*-acetylated sugars, suitable prey are selected according to the frequencies and amplitudes of their swimming movements. Appropriately sized prey stimulate hair bundle mechanoreceptors. Excitatory signals originating at the sensory neurons excite cnidocytes to discharge maximally into objects contacting the tentacle. The wounded prey releases proline into the seawater. Proline frequency tunes maximal discharge to higher frequencies and to larger amplitudes. Thus, struggling movements by the prey stimulate additional maximal discharge. Once the prey succumbs to nematocyst venoms, the prey is ingested.

Acknowledgment

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The photobiology of *Hydra's* periodic activity

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Key words: periodic behaviour, phase shift, photosensitivity, light wavelength, photopigments, opsin-like protein

Abstract

Hydra's response to a light pulse is a phase shift of the state of bioelectric activity correlated with the periodic shortening-elongation behaviour. The direction and absolute value of a phase shift depend on intensity, direction, application phase (along the periodic activity state), and wavelength of the light pulse. Repetitive pulses entrain the behavioural cycle. The period of the behavioural cycle depends on intensity and wavelength of steady background illumination; however, the light effect is not exerted isotropically along all the phases of the behavioural cycle. Inferences are drawn on light influence on the behaviour pacemaking mechanism. By using polyclonal antibodies against squid rhodopsin, an opsin-like protein has been identified in the ectodermal layer, presumably in sensory cells.

Introduction

Light stimulation has various steady or transient effects on the temporal patterning of *Hydra*'s periodic alternation between body-shortening and body-elongation phases. This behaviour is triggered, maintained, and modulated by a bidimensional nervous net that is structurally and functionally almost, but not totally, isotropic. Compound bioelectric events are correlated to the periodic movements. A series of 'contraction pulses' (CPs), i.e. a 'contraction pulse train' (CPT), arises at the beginning of each shortening phase, a series of 'rhythmic pulses' (RPs) at the beginning of each elongation phase. The pacemaking systems are located at hypostomal loci and at pedal and lower column zones, respectively, for CPs (transmitted mainly in the longitudinal direction) and RPs (transmitted mainly in the direction of body circumference) (Passano & McCullough, 1962, 1963, 1964, 1965; McCullough, 1965; Josephson, 1967; Josephson & Macklin, 1967; Shibley, 1969; Kass-Simon, 1972; Kass-Simon & Passano, 1978). A mutual inhibition is exerted between the two pacemaking groups (Passano & McCullough, 1965; Rushforth, 1971, 1973), probably as a post-inhibitory rebound of each group after the inhibition received by the other group (Taddei-Ferretti & Chillemi, 1987). A 'big slow wave' (BSW) is a periodic bioelectric activity of the whole organism with the same frequency as that of the periodic behaviour. Its trend is opposite at the two ends of the animal: the hypostome BSW is electrically more negative than the base BSW (the potential difference varies periodically with the above-mentioned frequency). CPs and RPs arise at fixed phases of the BSWs: CPs at the falling phase of the base BSW/rising phase of the hypostome BSW (when the potential difference between the two BSWs rapidly decreases), RPs at the opposite phase (when the potential difference smoothly increases) (Taddei-Ferretti et al., 1976, 1987).

Any bioelectric correlate of body movements may be used to monitor frequency variations of the periodic behaviour in response to light. Our landmark was the onset of the first CP of each CPT; we quantified a transient response as a phase shift (in direction of either advance or delay) of the

occurrence of the next expected landmark (by expressing the shift value as a fraction of the previously determined mean duration, normalized to 1, of the behavioural sequence of the tested animal in undisturbed conditions).

Responses to white light

The period of the behavioural cycle is shorter in darkness than in white light (Taddei-Ferretti et al., 1992). The periodicity of *Hydra* behaviour is not precise, but may be brought to a higher regularity (for a period up to 40× the behavioural period length) by a few light pulses (Taddei-Ferretti & Cordella, 1976) and even by a single one (Taddei-Ferretti & Musio, 1999).

After a light step of increasing light intensity (thereafter positive step) the behavioural period increases, after a step of decreasing light intensity (thereafter negative step) it decreases (Passano & McCullough, 1964; Borner & Tardent, 1971; Taddei-Ferretti & Cordella, 1976). After a positive light step, RPs are inhibited and then resume at higher frequency (Passano & McCullough, 1962), and the duration of the RP series and the CP frequency increase (Taddei-Ferretti et al., 1987).

The response to a light pulse is a phase shift of the state of bioelectric activity correlated with the periodic behaviour. A photic stimulus applied during a CP phase produces a phase shift to an RP phase; applied during an RP phase produces a shift to another RP phase (Passano & McCullough, 1962, 1963, 1964, 1965; Taddei-Ferretti & Cordella, 1975). The direction and the absolute value of the phase shift depend on the application phase of the light pulse (defined as the value of the phase – along the periodic bioelectric activity-states and related to the chosen landmark as phase zero – at which the pulse is applied) (which means that the latency until the occurrence of the next CP-landmark is not a constant), as well as on its intensity and direction (light pulse on darkness/darkness pulse on light) (Taddei-Ferretti & Cordella, 1976). At successive application phases of the pulse after the beginning of a CPT, decreasing advances of the next CPT are obtained for a light pulse and then increasing delays at further successive application phases, while decreasing delays, and then increasing advances, are obtained for a darkness pulse.

Consequences of the correlation between application phase of a light pulse and phase shift of the bioelectric activity state are (1) repetitive pulses entrain the behavioural cycle, while the phase relation between each pulse and each given behavioural event depends on the difference between event frequency and pulse frequency (Taddei-Ferretti & Cordella, 1975, 1976), and (2) repetitive light-pulse stimulation of a suitable frequency suppresses (as long as the stimulation continues) a given event of the behavioural sequence, producing a repetitive phase shift to the phase of a different behavioural event (Taddei-Ferretti et al., 1987, 1994a, b).

A light pulse localized at the base of the animal does not affect a CPT, but momentarily interrupts the RPs; localized at the hypostome does not affect the RPs, but definitively interrupts a CPT (Passano & McCullough, 1962, 1964).

The potential difference between the base BSW and the hypostome BSW is lessened for a while after a positive light step, and hightened after a negative step (Taddei-Ferretti et al., 1987). The dependence on application phase of a light pulse or darkness pulse is observed also for the effects on the base BSW and hypostome BSW (Taddei-Ferretti et al., 1976, 1987).

Responses to monochromatic light

Hydra is red blind (Passano & McCullough, 1963). The sensitivity to light of a given wavelength decreases if the animals are cultured under illumination at the same wavelength, and increases if they are cultured in darkness (Ellis, 1970).

The length of the behavioural period (inter-CPT period) varies inversely with the wavelength of the steady background illumination (Taddei-Ferretti & Cotugno, 1988; Taddei-Ferretti et al., 1992) but does not affect the inter-CP period, the inter-RP period, or the CP number of a CPT (Taddei-Ferretti et al., 1997). With various series of alternating light steps of two alternating different wavelengths, the greater duration of RP phases was obtained with short wavelengths (Tardent et al., 1976).

For a fixed application phase (and fixed intensity and duration) of a light pulse, the direction and the absolute value of the phase shifts depend on the pulse wavelength. For example, for an

application phase corresponding to the beginning of the CPT, when the absolute value of the response is maximum, a 550 nm pulse produces an advance of the next CPT, while for the same application phase a 450 nm pulse produces a delay (Taddei-Ferretti et al., 1988). For an application phase corresponding to the beginning of the RP series, a light step of longer or shorter wavelength produces respectively a longer or shorter latency until the next CPT (Singer et al., 1963).

The effect of two light pulses is not the sum of the two effects, but results from a non-linear interaction: (1) if two light pulses of different wavelenght and same intensity are applied at the same time (i.e. with the same application phase), the response value is intermediate between that obtained with the longer and shorter wavelengths alone (Taddei-Ferretti & Cotugno, 1991); (2) if the two stimuli are applied in close succession (i.e. with different application phases), the response value is shifted towards that of the second stimulus (Taddei-Ferretti et al., 1991).

For a fixed wavelength (and fixed application phase, intensity, and duration) of a light pulse, the shifts are not fixed (as expected, because the shifts are expressed in normalized units, as fractions of the mean behavioural sequence – determined at the same wavelength of steady background illumination as that during the pulse application – normalized to 1), but depend on the wavelength of steady background illumination (Taddei-Ferretti et al., 1992).

The period of the behavioural cycle at a fixed wavelength of steady background illumination increases with the decrease of the steady ambient temperature value (Taddei-Ferretti & Musio, 1994); however, the value of the above period increases is higher at lower background illumination levels (Taddei-Ferretti et al., 1997); thus the interaction between temperature and wavelength is not linear.

Cells and pigments responsible for photosensitivity

Rabbit polyclonal antibodies against squid rhodopsin were used on whole mounts of starved individuals of *Hydra vulgaris* and, for the first time, after immunofluorescence microscopy examination a rhodopsin-like protein was immunocytochemically identified and localized in the ectodermal layer. The number and distribution pattern of the fluorescent cells, as well as the presence of immunoreactivity in their axonal processes and ciliary appendages, speak in favour of sensory cell identification (Musio et al., 2001).

Discussion

The main results on *Hydra* photoresponses are (1) the difference of the photoresponses depending on the zone of the body that is stimulated (Passano & McCullough, 1962, 1964); (2) the strict dependence of the direction and the absolute value of the response on the application phase of a light pulse along the behavioural cycle (Taddei-Ferretti & Cordella, 1976); and (3) for a fixed application phase, their dependence on the light-pulse wavelength (Taddei-Ferretti et al., 1988). Inferences about the pacemaking mechanism may be drawn from photoresponses. (1) An asymmetry between the CP pacemaking system and the RP system may be inferred by the fact that, while a light pulse may stop an ongoing CPT, replacing it with an RP series, it inhibits the RP series only momentarily without replacing it with a CP one (Passano & McCullough, 1962, 1963, 1964, 1965), (2) The Rushforth (1971) hypothesis of a CPT generator different from a CP generator is confirmed by the fact that the inter-CPT period – but neither the inter-CP period nor the CP number of a CPT – depends on the wavelength of steady background illumination, and (3) The fact that, for a fixed application phase, intensity, duration, and wavelength of a light pulse, the phase shift is not fixed, but depends on the wavelength of steady background illumination (although the amount of the response is expressed in normalized units) (Taddei-Ferretti et al., 1992) means that the modulation of the two pacemaking systems of the periodic behaviour exerted by the steady illumination wavelength does not occur isotropically along all the phases. Thus, the limit cycle trajectory describing the phase state of the overall triggering system varies – not only in its size, but also in its shape – with the background illumination wavelength (Taddei-Ferretti & Musio, 1999).

The responses are elicited in spite of the fact that the photosensitive cells are not organized into an even very simple organ. Thus, it constitutes a

case of extraocular photoreception (Taddei-Ferretti & Musio, 2000; see also Yoshida, 1979). The candidates for the photoreceptive function may be either the epitheliomuscular cells or the neurons of the net (either the ganglion or the sensory cells). For a review of the various photoreceptor types in Cnidaria, see Martin (2002). At this point, two questions remained open: (1) which is the cell type responsible for the *Hydra* photosensitivity, and (2) which is (are) the functional visual pigment(s) responsible for phototransduction. For the first question, already Kass-Simon and Diesl (1977) recorded intracellularly a light effect in dissociated endodermal epitheliomuscular cells: the spontaneous oscillation frequency of the resting potential increased after a light pulse. In order to further analyze which are the candidates for photosensitivity, patch-clamp techniques were successfully applied to cells of *H. vulgaris* for the first time, in spite of the difficulties due to the very low ionic strength of the animal medium. Resting potentials were recorded in the whole-cell configuration and gigaseals were obtained from ectodermal epitheliomuscular cells enzymatically dissociated (Santillo et al., 1997). The next step should be to repeat the experiments in light-varying conditions.

To both the second and first question, the identification of an opsin-like protein in the ectodermal layer, presumably in sensory cells, is an answer (Musio et al., 2001). Opsin proteins have been found to be visual pigments in ocular and extraocular photoreception of vertebrates and invertebrates (Hara & Hara, 1980; Robles et al., 1986; Gartner & Towner, 1995; Kojima & Fukada, 1999; Yokoyama, 2000), and thus the identified rhodopsin-like visual pigment may be involved in *Hydra* photoresponses, constituting a clear case of neuronal extraocular photosensitivity (Musio et al., 2001), and confirming the Passano and McCullough (1963) hypothesis of a photoreceptive function of some neuronal cells of *Hydra*. Furthermore, works are currently in progress in order to isolate and amplify the *Hydra* visual pigment gene. We have designed pairs of oligonucleotide primers for PCR. The primer sequences correspond to some of the most conserved regions of a number of invertebrate and vertebrate published opsins (Santillo et al., 2002). Also a phylogenetic comparison with non-rod, non-cone photopigments (melanopsin, panopsin, peropsin, etc.) is underway (Foster & Hankins, 2002). This approach fits the new challenging field in vertebrate and invertebrate vision research represented by the searching for novel photopigments involved in the mechanisms of temporal and behavioural physiology regulated by extraretinal photoreception (Foster & Soni, 1998; Gao et al., 1999; Helfrich-Forster et al., 2002). A chemo- and/or mechanoreceptive function has been claimed for *Hydra* sensory neurons (Kass-Simon & Hufnagel, 1991). However, sensory neurons of Cnidaria, as well as their ganglion neurons, may be multifunctional sensory-inter-moto-neurosecretory neurons presenting from one to four such functions (Westfall, 1973; Westfall & Kinnamon, 1978; Grimmelikhuijzen & Westfall, 1995). A photoreceptive function of a ciliated sensory neuron conforms with the ciliary line of photoreceptors (Eakin, 1972), although recent studies on the identification of the gene Pax6 (a morphogenetic master gene discovered in the visual pigment of all animal phyla) in jellyfish and *Hydra* (Sun et al., 1997) could support the theory of the monophyletic origin of the various types of visual structures and suggest strong analogies between the elementary molecular mechanisms of different phototransductive pathways (Gehring, 2002).

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Photoreceptors of cubozoan jellyfish

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Abstract

The anatomically sophisticated visual system of the cubozoan jellyfish *Carybdea marsupialis* is described. Individual cubomedusae have eight complex eyes, each with a cornea, lens, and retina of ciliated photoreceptor cells, eight slit ocelli, and eight dimple ocelli. The photoreceptor cells of the complex eyes are bipolar and resemble vertebrate rod cells. Each photoreceptor has an outer cylindrical light-receptive segment that projects into a vitreous space that separates the lens and the retina, an inner segment rich in pigment granules, and a basal region housing the nucleus. The outer segment is a modified cilium with a 9 + 2 arrangement of microtubules plus stacks of membrane. These stacks of membrane form numerous discs that are oriented transversely to the long axis of the cell. The outer segment is connected to the inner segment by a slender stalk. The basal end of each photoreceptor forms an axon that projects into an underlying layer of interneurons. Each ocellus is composed of ciliated photoreceptor cells containing pigment granules. Rhodopsin-like and opsin-like proteins are found in the membrane stacks of the outer segments of the photoreceptors of the complex eyes. An ultraviolet-sensing opsin-like protein is present in the inner segments and basal regions of some of the photoreceptors of the complex eyes. Rhodopsin-like proteins are also detected in the photoreceptors of the slit ocelli. The cellular lens, composed of crystallin proteins, shows a paucity of organelles and a high concentration of homogeneous cytoplasm. Neurons expressing RFamide (Arg-Phe-amide) comprise a subset of interneurons found beneath the retinas of the complex eyes. RFamide-positive fibers extend from these neurons into the stalks of the rhopalia, eventually entering into the subumbrellar nerve ring. Vision may play a role in the navigation, feeding, and reproduction of the cubomedusae.

Introduction

Cnidarians have multicellular light detecting organs called ocelli (eyes). These photoreceptive organs include simple eyespots, pigment cups, complex pigment cups with lenses, and camera-type eyes with a cornea, lens, and retina (for a review, see Martin, 2002). Ocelli contain sensory photoreceptor cells interspersed among nonsensory pigment cells. Photoreceptor cells are bipolar, the apical end forming a light receptor process and the basal end forming an axon. A cilium with a 9 + 2 pattern of microtubules projects from the receptor-cell process. The membrane covering the cilium may show several variations, including evaginating microvilli. Pigment cells are rich in pigment granules, and in some animals the distal regions of these cells form tubular processes that project into the cavity of the ocellus. Microvilli may extend laterally from these tubular processes and interdigitate with the microvilli from the ciliary membranes of photoreceptor cells.

As cnidarians exhibit a diversity of eye designs, the evolution of these ocelli is fascinating (Singla, 1974; Eakin, 1982; Westfall, 1982). Photosensitive cells underwent morphological changes to produce

eye cups, eye cups with lenses, and complex eyes with a cornea, lens, and retina. These changes in eye pattern illustrate improvements of design wherein there is an increase in detectable spatial information (Gregory, 1967; Nilsson, 1989, 1990; Osorio, 1994). The ocelli of cubomedusae represent the most highly evolved eyes in the Cnidaria. In these ocelli, the opening of the eye cup constricted and a spherical, graded-index lens formed in the center of curvature of the retina, constructing a camera-type eye. Similar camera-type eyes, as seen in fish and cephalopods, achieve virtually aberration-free imaging over a full 180° visual field (Land & Fernald, 1992). The intricate visual system of a cubomedusa is reviewed in this paper, documenting that these animals do contain the optical-eye tools (opsins, crystallin proteins) required for photoreception and imaging.

Materials and methods

Jellyfish of *Carybdea marsupialis* Linnaeus were collected in the early afternoon in October 1998–1999 from shallow waters around Santa Barbara, California. Specimens were placed in one gallon ziplock plastic bags filled with seawater and transported to the lab. Medusae were placed in large finger bowls of seawater and observed and photographed using a Wild dissecting microscope. The stalks of the four eye-bearing structures, called rhopalia, were severed with a razor blade, thus freeing the rhopalia from the jellyfish bell. Larger pieces of the cubomedusae that included the rhopalia, their stalks, and surrounding bell tissue were dissected using fine scissors.

For immunocytochemistry, whole rhopalia and rhopalia with their stalks and pieces of surrounding bell tissue were fixed overnight at 4 °C in 4% paraformaldehyde in 0.2 M Millonig's buffer, pH 7.2, then washed 3× for 20 min each in 0.2 M Millonig's buffer, followed by 1 h in 0.4 M glycine, pH 7.2. Samples were again washed 3×, 20 min each, in 0.25% Triton X-100 in 0.1 M Millonig's buffer, and incubated overnight at 4 °C in a primary antibody diluted in 0.1 M Millonig's buffer containing 0.25% Triton X-100 plus 0.25% human serum albumin. Primary antibodies included affinity purified rabbit antisera raised against either blue-, green-, red-, or ultraviolet-sensing opsin proteins of zebrafish, rho-c (a rabbit polyclonal to the carboxyl terminus of zebrafish rhodopsin), rhodopsin 1 and rhodopsin 4 (rabbit polyclonals to *Drosophila* rhodopsins), Mab24B10 (a mouse monoclonal that recognizes the protein chaoptin in the membranes of the photoreceptors [larval and adult] of *Drosophila*), and RFamide antiserum 146 III (a rabbit polyclonal). Samples were washed 3× for 20 min each in 0.1 M Millonig's buffer containing 0.25% Triton X-100, followed by an incubation (1 h) at room temperature in either anti-rabbit FITC-IgG diluted 1:20 or anti-mouse FITC-Igs diluted 1:60 in 0.1 M Millonig's buffer containing 0.25% Triton X-100 and 0.25% human serum albumin. Specimens were rinsed 3× for 20 min each in 0.1 M Millonig's buffer and mounted on microscope slides using 50% glycerin in 0.1 M Millonig's buffer containing 5% n-propyl gallate. Whole mounts were examined and serially reconstructed with a BioRad MRC 1024 laser scanning confocal microscope.

For controls, rhopalia were also processed as described above except that the primary antibodies were eliminated. No staining was observed in these samples.

Results

General observations

Swarming cubomedusae of *Carybdea marsupialis*, a box jellyfish, appear seasonally during late summer and fall into January along the California coastline near Santa Barbara. This jellyfish has a clear bell, except for some small brownish flecks, giving it a slightly peppered appearance. Bell size ranges from 20 to 40 mm in height, with extended tentacles close to 1500 mm long. *Carybdea* is found in the shallow waters in the near shore part of the kelp beds known as sand channels. They are very patchy, however; when found, they are usually in densities of 1–2 over a 9 m^2. Densities of 30–50 m^{-3} have been reported in recent years. The jellyfish seem to stay in the sunny areas between the kelp beds over the clean sand. When they venture into the shadows of the kelp they reverse course and head back to the sunny areas. Shading them with a hand also results in their changing direction. They are positively phototactic and are

easily captured using night lights. Their average water depth is 4 m, where they are found within a meter of the bottom trailing their tentacles just above the sand. Here they feed on swarms of mysids and fish hatchlings.

Structure of the cubozoan visual system

Carybdea marsupialis has an anatomically sophisticated visual system (Martin, 2002). This cubozoan has four sensory structures, called rhopalia, one on each quadrant of the bell. Each rhopalium, measuring 500 μm in diameter, is a club-shaped structure suspended by a stalk, roughly 100 μm in length, from the bell. The rhopalia are in small indentations of the bell tissue; each indentation is covered by a v-shaped flap of tissue, similar to an eyelid. Each rhopalium has six eyes: two complex and four simple (Martin, 2002). The complex eyes, one small and one large, have a cornea, lens, and retina of ciliated photoreceptors. The large eye is directed laterally in toward the center of the bell (away from the outer v-shaped flap), and the small complex eye is directed upward toward the apex of the bell. The simple eyes, called ocelli, are located between the large and small eyes and also face inward. Two slit ocelli are found just above and on either side of the large complex eye, and two invaginated, dimple ocelli are found on either side of the small complex eye. The stalks of the rhopalia can twist and swing back and forth, resembling a pendulum of a clock. Thus the animal has an unobstructed view of its surroundings. The transparency of the bell allows each eye multidirectional vision, while the opening of the indentation may provide a clear field of photoreception in certain directions.

Complex eyes

The large eye is 350–400 μm in diameter with a lens measuring 150 μm in diameter, whereas the small eye is 250–300 μm in diameter with a lens diameter of 150 μm. The structure of the large complex eye will be described here. It consists of a cornea, spherical lens, and concave retina of ciliated photoreceptor cells (Fig. 1). The cornea covers the lens and is composed of a layer of flattened, squamous epithelial cells bearing numerous microvilli and an occasional short cilium. The

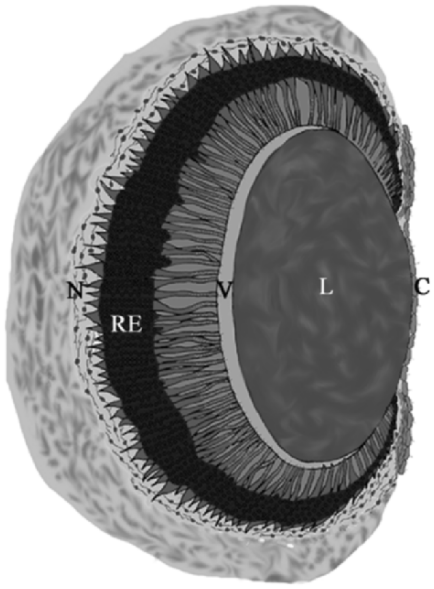

Figure 1. Diagram of the large complex eye of *Carybdea* illustrating the cornea (C), lens (L), vitreous space (V), retina (RE), and interneurons (N). Each photoreceptor consists of three parts: an outer cilium-bearing, light-receptive segment, filled with stacks of membranes that project into a vitreous space, an inner segment rich in pigment granules, and a basal region housing the nucleus. The base of the photoreceptor tapers to form an axon that projects into a plexus of interneurons located directly below the retina.

corneal epithelium is continuous with the epithelium of the rhopalium; the rhopalium epithelium contains cells bearing numerous microvilli and long cilia. Below the corneal epithelium is a heterogeneous, biconvex lens. The outer third of the lens is composed of stacks of fibrous cells while the center of the lens is defined by more globular to cuboidal-shaped cells. The central lens cells stain darker than do the outer lens fibers, indicating a greater concentration of proteins in the center cells. The lens fibers have a finely granular cytoplasm with a few mitochondria, polysomes, and profiles of endoplasmic reticulum. A small vitreous space separates the lens and the retina. This space is filled with a structureless, gelatinous mass. A layer of long, slender, closely packed photoreceptor cells, each 50–60 μm in length, comprises the retina of the eye. Each photoreceptor cell has an outer cylindrical light-receptive segment that projects towards the vitreous space. In the living state, the outer segment appears homogeneous and highly refractile under the light microscope.

Electron microscopy indicates that the outer segment is a modified cilium with a 9 + 2 arrangement of microtubules plus stacks of parallel lamellae (Martin, 2002). These stacks of membrane form numerous discs that are oriented transversely to the long axis of the cell. The outer segment of the photoreceptor cell is connected to an inner segment by a slender stalk which contains nine longitudinally oriented doublet microtubules that arise from a basal body in the distal end of the inner segment. A few cross-striated rootlets, originating from the basal body of the cilium, extend inward among pigment granules of the inner segment. The distal portion of the inner segment is rich in pigment granules, elliptical or rounded in shape. The alignment of the pigment granules in the inner segments of the photoreceptors produces a dark dense band that extends across the entire retina, giving the eye a dark color when viewed from the exterior. These pigment granules may shift in position along the length of the cell, depending on lighting conditions. Pigment granules have been observed in different locations in the photoreceptors (Martin, 2002). Microtubules are abundant in the cytoplasm surrounding the pigment granules. Nuclei of the photoreceptor cells are located in the basal regions of the cells. The base of each photoreceptor cell tapers and projects into a neural plexus of interneurons (nerve cells located just outside the ocellus) found directly below the retina. This plexus contains bipolar neurons, many of which contact neurons in the stalk of the rhopalium. Some of these stalk neurons send extensions into the main subumbrellar nerve ring of the bell of the jellyfish.

At least two types of photoreceptor cells are found in the large complex eye: numerous light-staining cells and an occasional interspersed dark-staining cell (Martin, 2002). The two types of cells have similar morphologies and contain pigment granules.

Ocelli

Two paired eye slits, each measuring 100 μm long, with rudimentary elongated lenses and two dimple ocelli (invaginated pits with a diameter of 25 μm) are lined with ciliated epithelial cells (Martin, 2002). These long, slender epithelial cells contain pigment granules similar in morphology to those detected in the complex eyes. The cells lining the dimple ocelli contain fewer pigment granules than do those of the slit ocelli. Further, not all cells lining the dimple ocelli contain pigment granules. Cell nuclei are located apically in these photoreceptors.

Rhodopsin/opsin staining

Three of the four visual organs exhibit positive staining with the rho-c antibody directed against zebrafish rhodopsin: the slit ocelli and the large and small complex eyes (Table 1, Figs. 2 and 3). In

Table 1. Cubozoan visual organs stained with rhodopsin/opsin antibodies (Ab)

	Large complex eye	Small complex eye	Slit ocelli	Dimple ocelli
Zebrafish Ab				
Rhodopsin	+	+	+	–
Red-sensing opsin	–	–	–	–
Green-sensing opsin	+	+	–	–
Blue-sensing opsin	+	+	–	–
UV-sensing opsin	+	+	–	–
Drosophila Ab				
Mab24B10 (Chaoptin)	–	–	–	–
Rhodopsin 1	–	–	–	–
Rhodopsin 4	–	–	–	–

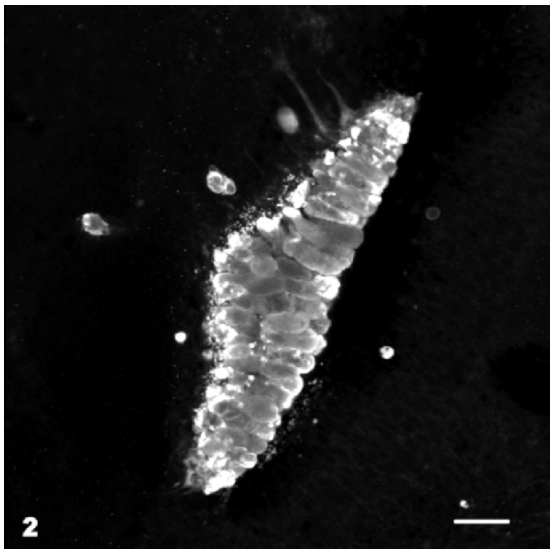

Figure 2. Slit ocellus stained with the antibody rho-c raised against zebrafish rhodopsin. The tips and bases of some of the photoreceptor cells are stained. Scale bar = 25 μm.

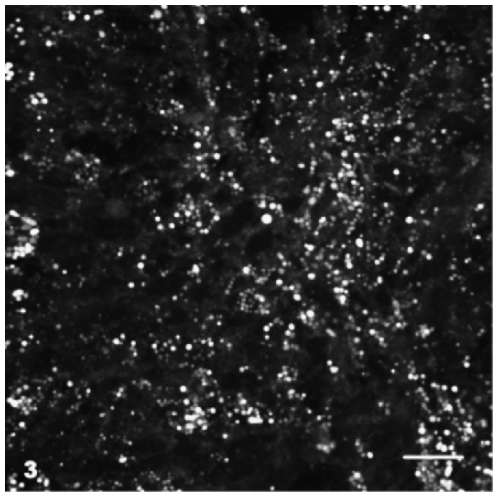

Figure 3. Rhodopsin-like proteins in the outer segments of the photoreceptor cells of the large complex eye. Scale bar = 13 μm.

the slit ocelli rhodopsin staining was visualized at the tips and bases of some of the photoreceptors (Fig. 2). In the complex eyes antibody staining was detected in the retina in the outer segments of some of the photoreceptor cells (Fig. 3). Small dots of stains were associated with the stacks of parallel membranes forming the discs in the outer segments. No staining was detected in the inner segments or basal regions of the photoreceptors. The dimple ocelli exhibited no labeling with the antibody. When antisera that detected blue-, green-, red-, or ultraviolet-sensing opsin proteins in zebrafish were applied to cubozoan visual organs, positive staining was seen for blue-, green-, and ultraviolet-sensing opsins in the large and small complex eyes (Figs 4, 5, 7 and 8). Antisera for red-sensing opsin proteins showed no binding to any of the visual organs (Fig. 6). The blue- and green-sensing opsin-protein staining was found in the outer segments of some of the photoreceptors of the retina (Figs 4 and 5). Small dots of stain were observed in the regions of the stacks of disc membranes. No staining was seen in the inner segments or basal areas of the photoreceptors. Staining for ultraviolet-sensing opsin proteins was detected in some of the photoreceptors of the retina (Figs 7 and 8). Unlike the staining for the blue-sensing and green-sensing opsins, the staining for ultraviolet-sensing opsin proteins was detected in the inner segments and basal regions of some of the photoreceptors. The ultraviolet-sensing opsin-labeled photoreceptors of the large eye and small eye showed very different morphologies (Figs 7 and 8). In the large eye, the stained photoreceptors were long, skinny spindle-shaped cells. Punctate staining was detected in the inner segments near the pigment granules and in the basal regions near the nuclei. Staining in the small eye was more

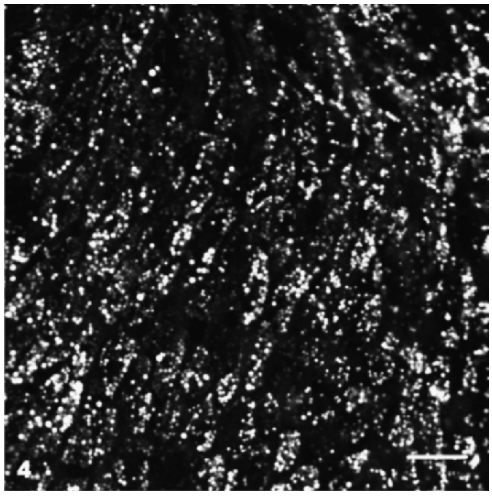

Figure 4. Outer segments of photoreceptor cells of the large complex eye labeled with antisera that detect the blue-sensing opsin protein of zebrafish. Scale bar = 13 μm.

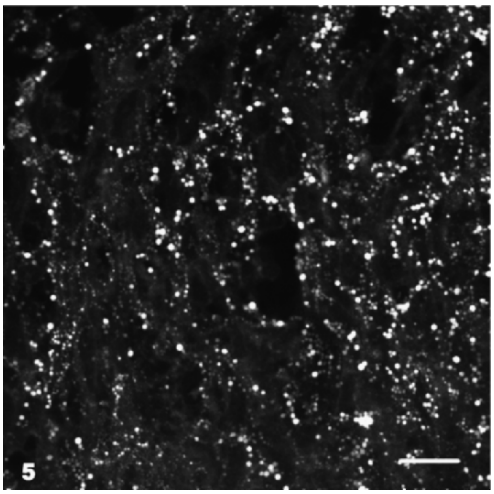

Figure 5. Outer segments of photoreceptor cells of the large complex eye labeled with antisera that detect the green-sensing opsin protein of zebrafish. Scale bar = 13 μm.

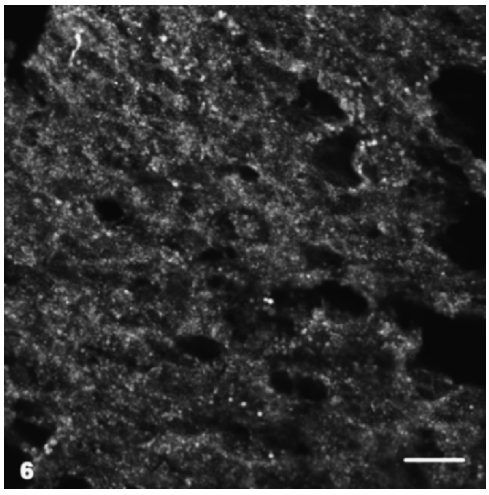

Figure 6. Outer segments of photoreceptor cells of the large complex eye labeled with antisera that detect the red-sensing opsin protein of zebrafish. Note the absence of staining. Scale bar = 13 μm.

intense than in the large eye and the labeled photoreceptors were globular in shape. The label was found in close proximity to the pigment granules in the inner segments. For both the small and large complex eyes the great majority of the ultraviolet-sensing opsin-labeled photoreceptors were located in the central part of the retina directly behind the lens. Staining tapered off and disappeared in photoreceptors located farthest from the center of the retina. In all cases where rhodopsin-like and

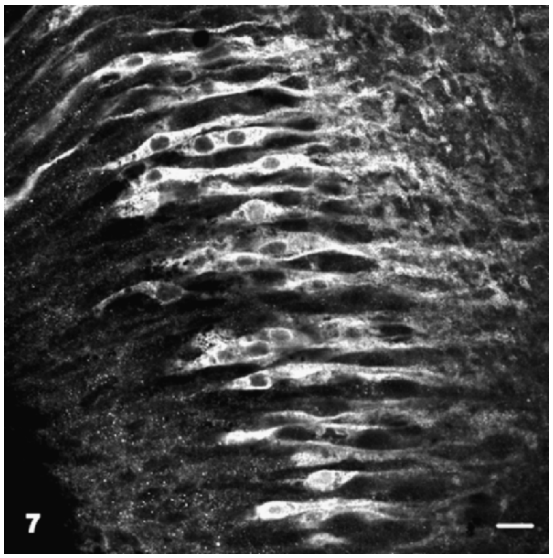

Figure 7. Photoreceptors of the large complex eye stained with antisera that detect ultraviolet-sensing opsin protein of zebrafish. These labeled photoreceptors show a very different morphology from the labeled photoreceptors of the small eye (shown in Fig. 8). Not all the photoreceptors of the complex eyes stain for the ultraviolet-sensing opsin protein. Scale bar = 25 μm.

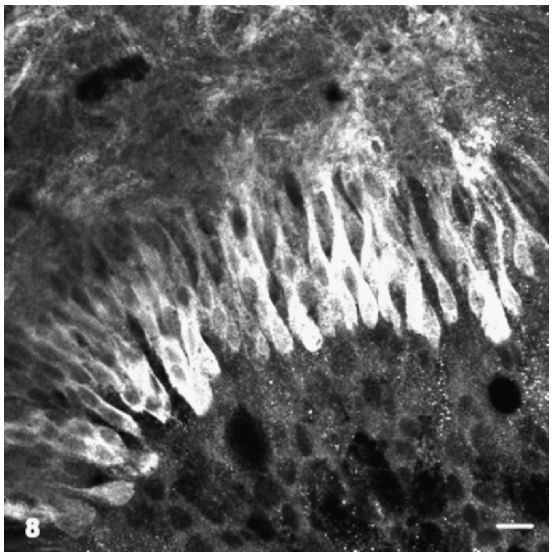

Figure 8. Photoreceptors of the small complex eye stained with antisera that detect ultraviolet-sensing opsin protein of zebrafish. Scale bar = 25 μm.

opsin-like proteins were detected in cubomedusae, the antibody labeling was restricted exclusively to photoreceptor cells. Cubozoan visual organs ex-

posed to antibodies made against *Drosophila* chaoptin (Mab24B10) and *Drosophila* rhodopsins showed no staining (Table 1).

RFamide staining

Cells expressing RFamide (Arg-Phe-amide) are found in the slit ocelli, in the neuronal layer located directly beneath the photoreceptors of the large and small complex eyes, and at the top of the rhopalium where the stalk is connected (Figs 9, 10 and 13). Further, fibers of neurons expressing RFamide project away from these complex eyes into the stalks of the rhopalia, pass up the stalks, and eventually enter into the subumbrellar nerve ring of the jellyfish (Figs 11 and 12). Such fibers may constitute part of the ocular nerves.

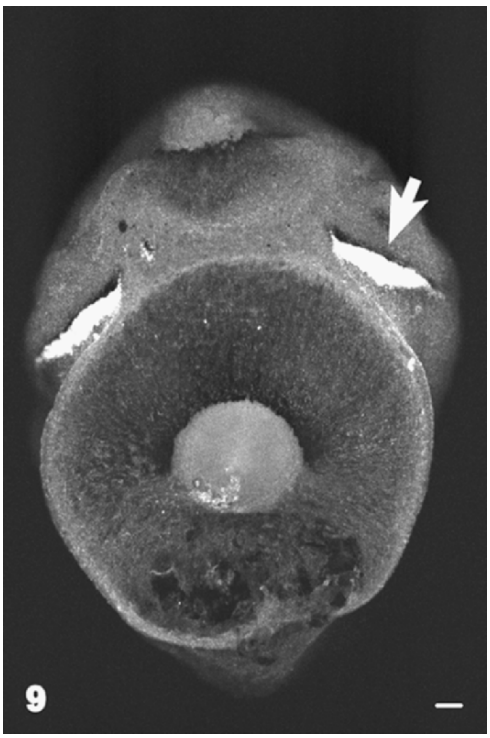

Figure 9. Whole-mount of a rhopalium labeled with antibodies directed against the neuropeptide RFamide. RFamide-positive cells are found in the slit ocelli (arrow), beneath the retina of the large and small complex eyes (Fig. 13), in the region of the rhopalium that connects to the stalk (Fig. 10), in the stalk (Fig. 11), and in the subumbrellar nerve ring (Fig. 12). Scale bar = 20 μm.

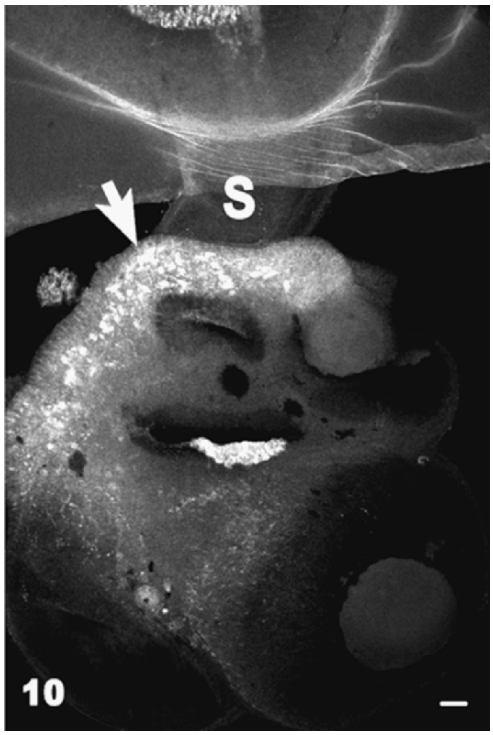

Figure 10. RFamide-positive neurons (arrow) in the region of the rhopalium that connects to the stalk (S). Scale bar = 20 μm.

Discussion

Cubomedusae have complex camera-type eyes that are optically competent. Further, they have the visual machinery required to form images. The lens, composed of novel crystallin proteins, is a graded-index lens in which the refractive index gradually changes across the lens, causing light to bend continuously toward the area of higher refractive index (Piatigorsky et al., 1989; Mackie, 1999; Martin, 2002). The center of the lens has the highest refractive index, gradually decreasing toward the periphery. Spherical aberration can be eliminated completely with a graded index lens, and the lens has a short focal length when compared to a homogeneous lens (Nilsson, 1990). Behind the lens is a small vitreous space. The photoreceptors of the retina extend their expansive light-receptive surfaces into this vitreous space. Such receptive surfaces contain numerous membranes derived from cilia that are arranged in stacked, disc-like structures oriented transversely

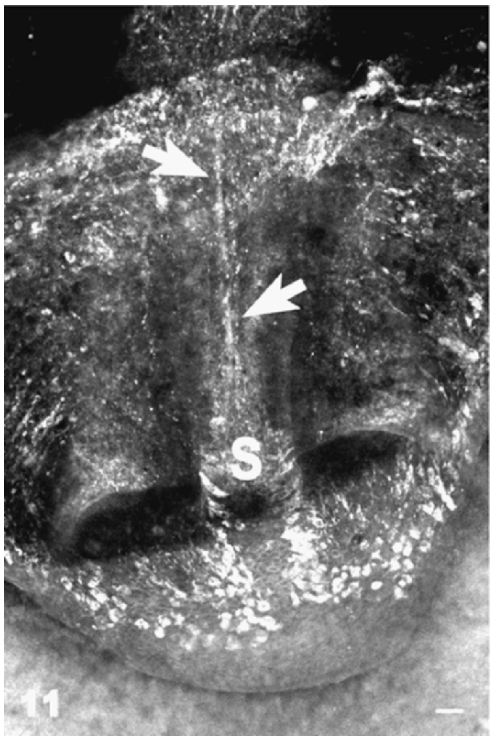

Figure 11. RFamide-positive neurons (arrows) in the stalk (S) of the rhopalium. Scale bar = 20 μm.

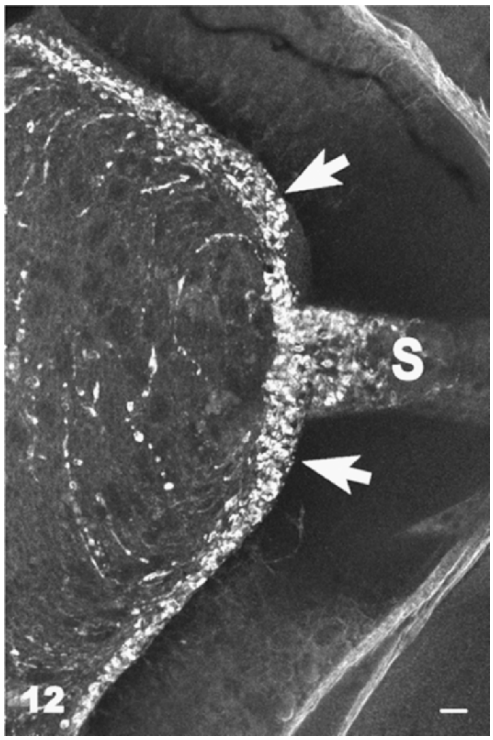

Figure 12. RFamide-positive neurons in the subumbrellar nerve ring (arrows). S – stalk. Scale bar = 20 μm.

to the long axis of the cell, similar to the stacks of membranes found in vertebrate rod cells (Fawcett, 1994). These discs are rich in rhodopsin-like and opsin-like proteins, the visual pigments involved in phototransduction. The photoreceptors contain pigment granules that may move in response to changing light conditions, most likely allowing the eye to adjust its sensitivity according to changes in ambient light (Martin, 2002). Also, the basal ends of the photoreceptors project into a plexus of interneurons; some of these neurons express RFamide, glutamate, serotonin, and GABA (Martin, 2002). These neurotransmitters are known to function in the photobiology of vertebrates and invertebrates (Lam et al., 1982; Lin et al., 2001).

As a competent image-forming eye would require visual processing, the question is asked: where would visual processing occur in cubomedusae? The nervous system of cubomedusae is complex and consists of a diffuse synaptic nerve net throughout the bell connected to a subumbrellar nerve ring (Satterlie, 1979). This subumbrellar ring consists of hundreds of neurons, some of which are giant neurons, that run circularly in the ring. Nerve bundles pass from the subumbrellar ring into the subumbrellar nerve net. Interneuronal synapses occur throughout the animal. The rhopalia are interconnected by the subumbrellar nerve ring, as there are four radial ganglia located where the nerve ring branches to connect to the rhopalia (Conant, 1898; Laska & Hundgen, 1984). The rhopalia contain nerve cells and their fibers, many of which synapse with interneurons located directly below the complex eyes. The photoreceptors of the complex eyes have basal processes that insert into a plexus of neurons formed by these interneurons. As cubomedusae have their eyes positioned radially around the margin of the bell, they can receive visual information from all directions. Further, the rhopalial ganglia, the proposed integrative centers for the eyes, are concentrated near the eyes. For a radially symmetrical animal, this radial arrangement of sensory structures and ganglia is critical for sensing the environment and for responding appropriately. The nerve rings of cubomedusae represent a maximum degree of neuronal localiza-

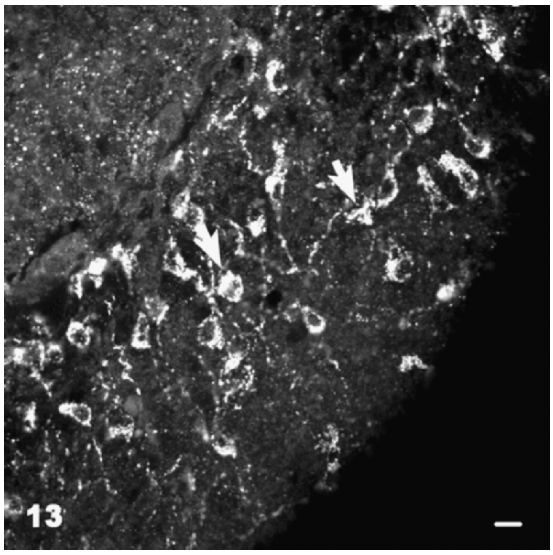

Figure 13. RFamide-positive neurons (arrows) beneath the retina of the large complex eye. Scale bar = 10 μm.

tion within the cnidarians, and the ring probably functions as the central nervous system of the jellyfish. Hence, visual information would be processed in this ring.

The complex eyes of cubomedusae may function in navigation, feeding, and reproduction. Cubomedusae are neritic, usually tropical or subtropical, and are commonly found in productive near-shore habitats such as sandy beaches, kelp forests, mangroves, and coral reefs (Werner, 1973). They are positively phototactic, agile swimmers, and voracious feeders (Barnes, 1966; Larson, 1976; Martin, 2002). Navigating in near-shore habitats is tricky, especially for soft bodied animals that are easily ripped apart by barriers in the environment. Spatial vision may allow the cubomedusae to freely move in their habitats, thus avoiding collisions with barriers. For example, the cubomedusa *Chironex fleckeri* navigates accurately between pier pilings and takes evasive action when people venture too close (Hamner, 1994). Many cubomedusae chase, catch, and eat fish (Larson, 1976). Further, some cubomedusae engage in copulation during mating (Studebaker, 1972; Werner, 1973; Stewart, 1996; Martin, 2002). Male medusae chase female medusae, grab female medusae with their tentacles, and inject spermatophores onto the tentacles of the females. Such sophisticated feeding and reproductive behaviors of cubomedusae are probably mediated by the complex eyes.

Rhodopsin-like and opsin-like proteins are found in the photoreceptor cells of cubomedusae and in some of the sensory nerves of hydroid polyps (Martin & Givens, 1999; Musio et al., 2001; Martin, 2002). In cubomedusae these proteins were visualized using antisera raised against vertebrate zebrafish opsins, and staining was restricted exclusively to photoreceptor cells. Further, the photopigment staining patterns of the cubozoan complex eyes suggest that the jellyfish have blue, green, and ultraviolet wavelength receptors and imply that the jellyfish, like many other invertebrates, can distinguish different wavelengths of light (Goldsmith, 1990).

Opsins found in the eyes of mollusks, arthropods, and vertebrates show sequence and structure conservation, indicating a common origin of the photoreceptor molecule (Gould, 1994; Wolken, 1995). The demonstration of opsin-like proteins in association with distinct photoreceptors of the complex eyes and ocelli of cubomedusae using antibodies raised against vertebrate opsins suggests some structure/sequence commonalities of these jellyfish proteins with those of vertebrates. Recently, an opsin gene was found among the Hydra EST sequences (R. Steele, personal communication). Hence, the origin of the opsins appears to date back to the cnidarians, the most primitive present-day animals displaying eyes.

Acknowledgments

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A mechanosensory system that controls feeding in adult *Mnemiopsis*

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Key words: food groove, cilia, sensory reception, vibration reception

Abstract

We describe here the food groove complex and mechanism of prey capture used by adult *Mnemiopsis* spp. ctenophores to obtain prey swept into the auricular grooves by feeding currents. Tentilla that emerge from the tentacular groove of the food groove complex extend into the auricular grooves and capture prey upon their sticky surfaces. The prey-laden tentilla contract and drag the prey to the edge of the transport groove, which is also part of the food groove complex. The transport groove undergoes a focal eversion to capture and transport prey orally. Focal eversion exposes the inner ciliated surface of the transport groove as it extends toward the prey. Focal eversion can be evoked by mechanical stimuli from a probe, but only if it is positioned directly over the tentacular groove. We propose that g-cilia located within the tentacular groove are mechanoreceptors whose output triggers a sensory-motor pathway that in turn everts the transport groove. The mechanosensory-motor pathway is ectodermal and sensitive to Mg^{2+} anesthesia, which defocuses and amplifies eversion. Tentilla are not strictly necessary for eversion to occur, because preparations lacking tentilla can still display eversion; however, they may amplify the sensory signal by interacting with g-cilia as they contract.

Introduction

Adult *Mnemiopsis leidyi* and *M. mccradyi* have four auricular grooves positioned between the body proper and the lateral extension of the feeding lobes. Four tentacular grooves (two on each side) extend laterally away from each of two small tentacular pouches located near the mouth. The tentacular grooves pass over the oral-most extension of the auricular groove and continue onto its lateral wall (Main, 1928) where they join up with an extension of the edge of the mouth referred to as the transport groove. Each tentacular groove is thus immediately medial to a muscular transport groove (Fig. 1). Sticky tentilla (fine tentacles) extend from the tentacular groove into the auricular groove, where they are swept aborally or medially by feeding currents generated by the auricular comb plates. Prey captured by tentilla are pulled close to the transport groove, which envelops the prey and transports it orally (Moss et al., 2001).

Close examination of the tentacular grooves revealed the presence of toothlike structures (Moss et al., 2001). The toothlike structures were named 'g-cilia' after 'Gemshorn Förmige Cilien' (goatshorn-like cilia), a name given to similar structures seen in *Cestum* ~120 years ago (Chun, 1880). Tentacular grooves contain a staggered single row of outer g-cilia and from 3 to 12 rows of inner g-cilia (Fig. 2).

Although there have been numerous examinations of the capacity and mode of feeding, we do not understand the physiology of prey capture by *Mnemiopsis*. This is remarkable considering the broad distribution of this genus and its importance to the coastal ecology of the Gulf of Mexico and

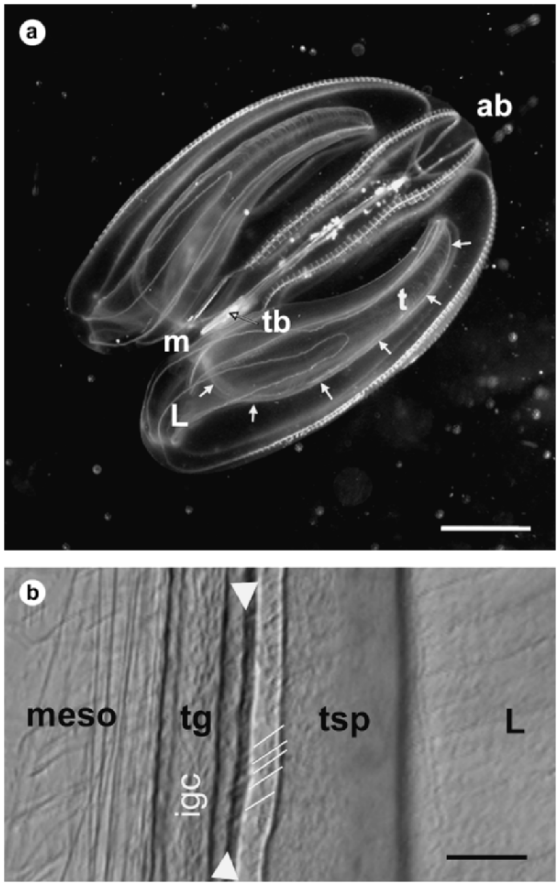

Figure 1. (a) *Mnemiopsis mccradyi*. Small white arrows indicate the position and extent of one of the food groove complexes on the lateral aspect of the auricular groove. The opposite side is left unlabelled for clarity. Many fine tentilla (t) extend into the auricular groove, which occupies the same region in the image as the tentilla. ab, aboral end of animal; m, mouth; tb, tentacular bulb; L, lobe. Scale: 5 mm. (b) The food groove complex. The mesoglea is medial; the lobe is lateral in this image. meso, mesoglea; tg, tentacular groove; tsp, transport groove; L, lobe; igc, inner g-cilia – visible as 'cobbles' on a path; fine white lines indicate position of outer g-cilia, located just off lower left tips; heavy white arrow heads indicate embedded tentacle within the tentacular groove from which the tentilla emerge. Scale: 50 μm.

the Atlantic seaboard, and the impact it has had in exotic environments (Kideyş, 2002).

Preliminary ultrastructural analyses of the g-cilium cell are tantalizing in that they suggest several different potential modes of function. The g-cilium itself is nonmotile, yet contains up to 50 conventional '9 + 2' cilia. Each cilium is surrounded by a lateral extension of an apical cytoplasmic protrusion. The cytoplasm is osmiophilic and contains mitochondria, bacteria, and numerous fine cross-linked filaments decorated by clear-core vesicles (Moss et al., 2001).

G-cilia vesicles may be part of a secretory system involved in toxin delivery (Moss et al., 2001); however, we have not observed omega bodies (indicative of secretion) in the plasma membrane. Vesicles may carry g-cilia structural components. Light microscopy, and transmission and scanning electron microscopy of g-cilia display a submicron tip (Moss et al., 2001; cf. Fig. 2). Thus, sharp g-cilia might induce autotomy in the tentilla, which detach readily when disturbed.

G-cilia are probably mechanoreceptors. Not only do g-cilia cells bear multiple cilia, but the cilia are anchored by particularly long and massive rootlet fibers. Thus, g-cilia are probably very stiff, like all known mechanoreceptors. Stiffness allows transmission of a mechanical disturbance to deform the somatic membrane in protists and invertebrates and so generate the bioelectrical signal (Stommel et al., 1980; Machemer & Machemer-Röhnisch, 1984; Machemer-Röhnisch & Machemer, 1984).

In this report we describe prey capture behavior by the food groove complex, and demonstrate mechanosensitivity in the tentacular groove of *Mnemiopsis*; thus, g-cilia are very close to the region of mechanosensitivity. We describe an initial pathway analysis. We also describe a model for the sensation and processing of mechanical stimuli.

Materials and methods

Ctenophores

Mnemiopsis mccradyi were collected by dipping at several sites from the northern Gulf of Mexico. Care was taken to not injure the animals. The ctenophores were transported to the Auburn University campus (~5 h drive), where they were placed in plankton kreisels for holding. Ctenophores were fed on alternate days with *Artemia salina* nauplii. Although *M. mccradyi* could be routinely held for several months in our lab, for the studies described here, they were used within a week of capture.

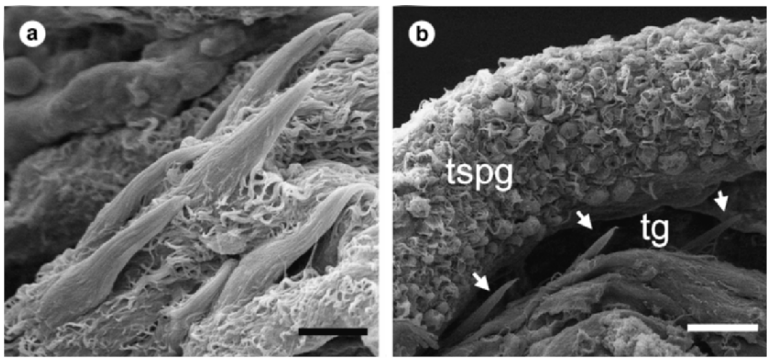

Figure 2. Scanning electron microscopy of g-cilia. (a) Inner g-cilia, scale: 5 μm. (b) Outer g-cilia just inside tentacular groove, tg, (arrows). tspg, everted transport groove, scale: 10 μm.

Specimen preparation for mechanical stimulation

Ctenophores either were used intact or were microsurgically dissected to produce reduced preparations. Reduced preparations consisted of the food groove complex, the lateral aspect of the auricular grooves, and associated lobe flaps. Excess mesoglea was removed.

Intact animals or reduced preparations were immobilized with the auricular groove up, which positioned the food groove complex so that it was accessible to micromanipulation. Preparations were immobilized with fine minuten pins or cactus spines (*Opuntia* spp.) that did not interfere with eversion.

Glass borosilicate or stainless steel microprobes were attached to a piezoceramic bimorph wafer held in a micromanipulator. Power supplied by a function generator caused the bimorph to bend, thereby moving the probe.

Video microscopy

Microscopy was carried out with a dissecting microscope equipped with a video port. Video passed through a signal processor for improved contrast, and was recorded on videotape. Video images were digitized with a capture board and software. For mechanosensory mapping, we used a special effects generator to inset an image of the oscilloscope on the image of the preparation so that we could simultaneously observe a record of the stimulus, the probe position, and the correlated muscular response.

Results

Mechanism of feeding

Artemia nauplii elicited identical feeding behavior in both intact animals and reduced preparations. A single tentillum could ensnare and drag prey close to the transport groove. The transport groove then everted immediately lateral to the prey-bearing tentillum. A single precisely directed eversion was sufficient to capture prey. The capture of a nauplius can be seen in Figure 3. Captured prey were transported to the mouth at 281 ± 17 μm s^{-1} ($n = 22$) in the auricular groove but at 422 ± 15 μm s^{-1} ($n = 40$) in the curve near the mouth.

Mechanosensitivity and eversion

We applied small amplitude mechanical stimuli to intact animals and reduced preparations to map mechanosensitivity. A probe provided stimuli to one of the following: inner lobe, auricular groove, tentilla, transport groove, or tentacular groove.

A gentle tap near the margin of a lobe evoked formation of a cuplike structure similar to bell margin crumpling in cnidaria. Tapping the lobe had no effect on the transport groove. Tapping the auricular groove lateral wall, the inner surface of the lobe near the food groove, or within the transport groove, did not evoke eversion, which was seen only if the probe tapped directly over the tentacular groove. A single tap caused a single eversion (Fig. 4) precisely directed toward the

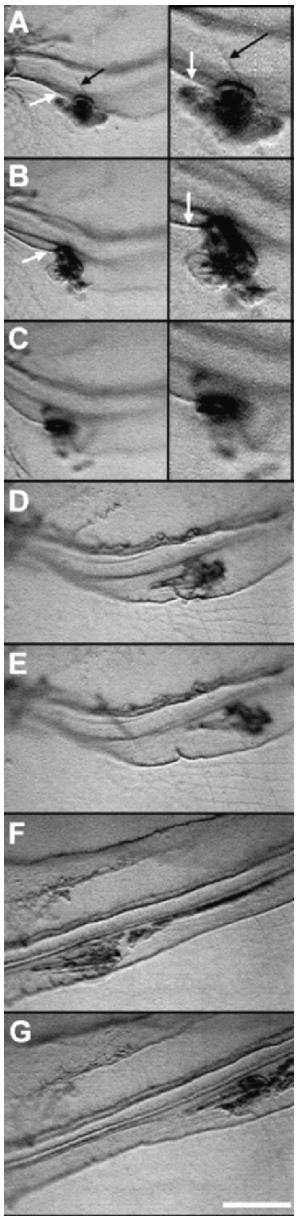

Figure 3. *Artemia* capture. Inset images to the right of (A)–(C) are magnified versions to show details. (A) Note tightly stretched tentillum (black arrows). (B) Eversion of transport groove (edge indicated by white arrows) and capture of prey. (C) Prey captured and enveloped in transport groove; out of focus indicates movement downward into the groove. (D) and (E) Orally-directed movement toward the right compared to position in (A)–(C). (F) and (G) Images taken from a different region closer to the mouth, showing orally directed movement of prey along transport groove. Scale: 100 μm: applies to (A)–(C) (left) and (D)–(G).

probe. See Table 1 for a summary of responses to tapping stimuli.

Vibrational stimuli applied very close to the tentacular groove also evoked transport groove eversions. Optimal stimuli were between 20 and 60 s^{-1}. Figure 5 shows an eversion in response to

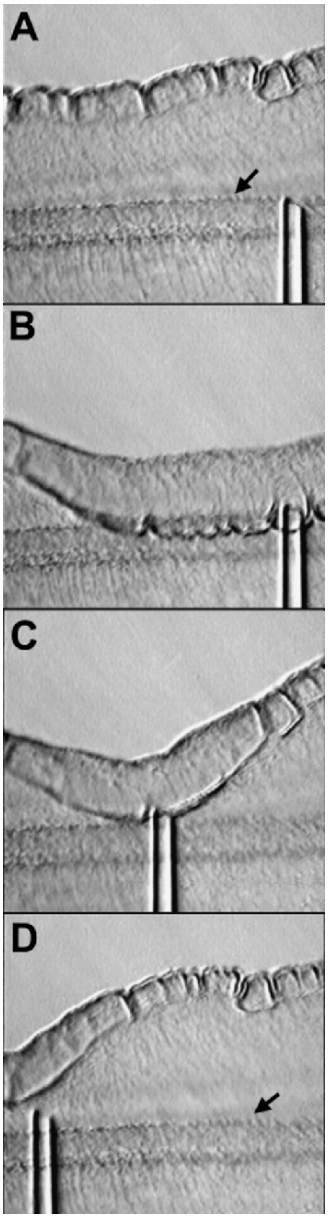

Figure 4. Eversion in response to single taps applied ~250 μm apart. (A) Prior to stimulus (B)–(D). Three different positions showing mechanosensitivity and transport groove eversion in response to a single tap. Small black arrows (A, D) indicate barely visible outer g-cilia.

Table 1. Eversions evoked by single taps

Location	No response	Eversion
Inner lobe surface	3	
Transport groove	5	
Tentacular groove		8
Tentilla		4
Auricular groove	5	

Values refer to the number of preparations.

vibration. Table 2 summarizes the responses to vibrational stimuli.

Tentilla were very sensitive to microsurgery and often autotomized; thus, they were not present in all reduced preparations (cf. Fig. 1(B)). Tentillar autotomy did not prevent eversion.

Mg^{2+} sensitivity

Intact ctenophores and reduced preparations relaxed under magnesium anesthesia in 25–29 ppt sea water containing ~56 mM added $MgCl_2$. The lobes ceased to move and subectodermal muscles ceased to contract in response to mechanical disturbance, although comb plates continued to beat. Even so, magnesium-induced anesthesia did not block eversion. Instead, mechanical stimuli evoked

Table 2. Eversions in response to vibrational stimuli

Frequency (s^{-1})	No response	Eversion
5	1	1
10		2
15	1	2
20		3
25		4
30		1
50		2
60		2

Values: number of preparations.

repeated transport groove eversion at multiple locations over the entire length of the food groove (Fig. 6). Eversions became more frequent with time, even occurring in the absence of stimulation. Replacement of the $MgCl_2$-enriched sea water with normal sea water restored normal behavior.

Microsurgical blockade

Very shallow microsurgical cuts made with an iridectomy knife, between and parallel to the tentacular and transport grooves, entirely ablated eversion over that region. Examination of such preparations by high magnification differential

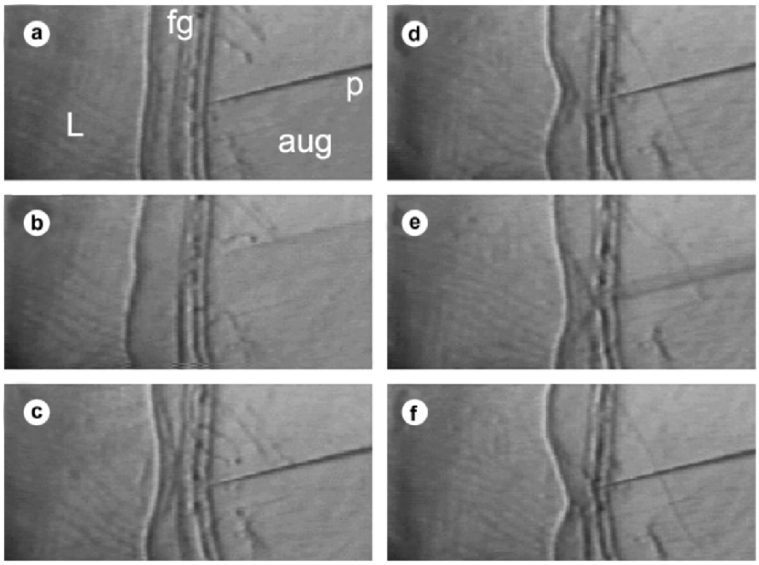

Figure 5. Oscillatory stimulus at 25 s^{-1}. Glass probe stimulus. (a) Prior to stimulus. (b) During mechanical stimulus (probe is blurred). (c)–(e) 'Capture' of probe (probe deflected out of focus in (e). (f) Relaxation, post-stimulus, probe is released. Approximately 5 s between images. L, inner surface of lobe; fg, food groove complex with transport groove to left; aug, auricular groove (cf. Fig. 1); p, probe.

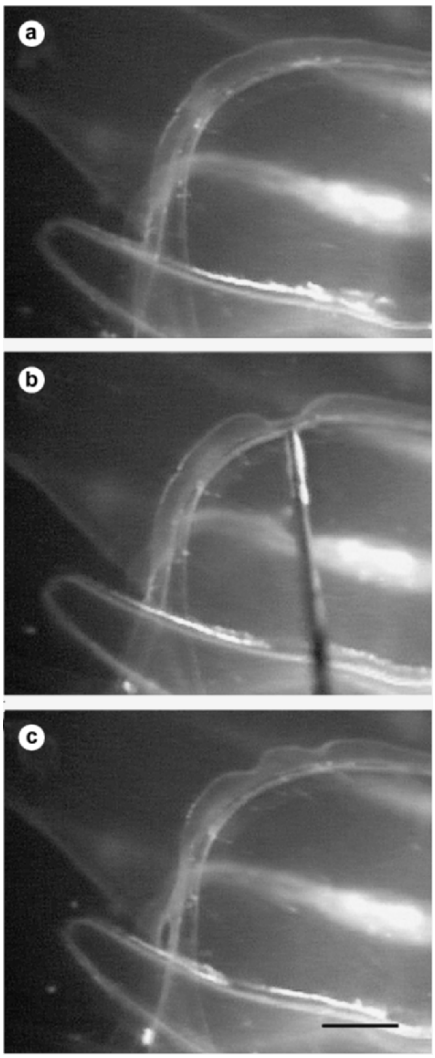

Figure 6. $MgCl_2$ anesthesia. (a) No eversion. (b) Response to a single touch to the tentacular groove. (c) Multiple eversions in the absence of stimulus. Dark field. Scale: 100 μm.

interference contrast showed that the subectodermal muscles were not cut. Eversion was unaffected beyond the ends of the cut, suggesting that the pathway passed linearly from the tentacular groove to the transport groove.

Discussion

Mnemiopsis *feeding*

Mnemiopsis is generally accepted to be a critical player in the trophic structure of the coastal zone (Reeve et al., 1989; Cowan & Houde, 1993; Purcell et al., 1994, 2001), and has been blamed for the collapse of the Black Sea and Caspian Sea fisheries (Khoroshilov & Lukasheva, 1999; Kideyş, 2002; Stone, 2002). Thus, there is a strong ecological imperative for mechanistic studies of *Mnemiopsis* feeding. Indeed, *Mnemiopsis* feeding mechanisms have attracted the attention of researchers for some time (Main, 1928; Baker & Reeve, 1974; Larson, 1988; Costello et al., 1999). Waggett & Costello (1999) reported that the inner surfaces of the oral lobes captured relatively large prey, such as adult copepods, while the food groove (here called the food groove complex) captured small prey.

While ctenophore neural connections are readily identified ultrastructurally (Hernandez-Nicaise, 1973), studies that effectively correlate the anatomy and physiology underlying common behaviors such as feeding (e.g. *Pleurobrachia*, Moss & Tamm, 1993) are very rare. To date, a clear anatomical correlate for a ctenophore neurociliary pathway has been shown only for *Euplokamis* (Mackie et al., 1992).

A mechanosensory system that controls feeding in Mnemiopsis mccradyi

We have described here a feeding response – transport groove eversion – evoked by mechanical stimulation. The region of mechanosensitivity – the tentacular groove – is easily accessible to manipulation. Single taps and vibrations both evoke transport groove eversion. The sensory-motor pathway appears to pass laterally from the tentacular groove to the transport groove within the ectoderm in what appears to be a straight line, leading to a focal eversion that captures prey.

Known mechanoreceptors and g-cilia

Cilia and flagella are inherently mechanosensitive (Thurm, 1968; Stommel et al., 1980; Tamm, 1982; Eshel et al., 1990). Cilia are often combined with actin-rich processes in specialized mechanosensors. Vertebrate hair cells combine a cilium – the kinocilium – with dense, actin-filled stereocilia, which are specialized microvilli (Howard et al., 1988), while cubozoa (Goltz & Thurm, 1993), capitate hydropolyps (Holtmann & Thurm, 2001), and anthozoa (Watson et al., 1997) all have very similar mechanoreceptors that combine cilia with

actin-rich microvilli. Tamm & Tamm (1994) reported presumed epithelial mechanoreceptors on the lips of beroid ctenophores that combined a single cilium anchored by an 'onion body' style basal body (Hernandez-Nicaise, 1974) with a spikelike, actin-filled 'Taststift'.

G-cilia fit the above structural motif because preliminary labeling studies in our laboratory indicate that the fine filaments found in the g-cilium cytoplasmic protrusion (Moss et al., 2001) are F-actin. Our operating hypothesis is that g-cilia are mechanosensors.

Lateral inhibition may produce focal eversion

Magnesium chloride anesthesia, which should block Ca^{2+}-dependent synaptic transmission, results in loss of focal eversion. This unexpected result strongly suggests that an important component of the mechanosensory pathway is inhibitory and blocked by Mg^{2+}. A lateral inhibition mechanism dependent upon conventional chemical neurotransmission may exist between mechanoreceptors or interneurons in the nerve net pathway. Even so, eversion persists and grows with continued exposure to $MgCl_2$ anesthesia, until it occurs even in the absence of overt mechanical stimuli. The lack of Mg^{2+} sensitivity in the motor pathway suggests that either there is no synapse between the mechanoreceptors and the muscles, or that there is an alternate conducting mechanism, such as gap-junction mediated epithelial conduction.

Tentilla as possible signal amplifiers

Tentilla are not essential to mechanosensation, because eversion occurred in their absence. Tentilla pass over g-cilia as they emerge from the tentacular groove. They probably come in contact with the g-cilia, particularly during prey capture. If g-cilia are mechanoreceptors, tentilla may amplify the signal from struggling prey by applying pressure to g-cilia.

A model of the mechanosensory system

A model is presented as a basis for further study (Fig. 7). The mechanosensory response should be proportional to stimulus proximity and amplitude.

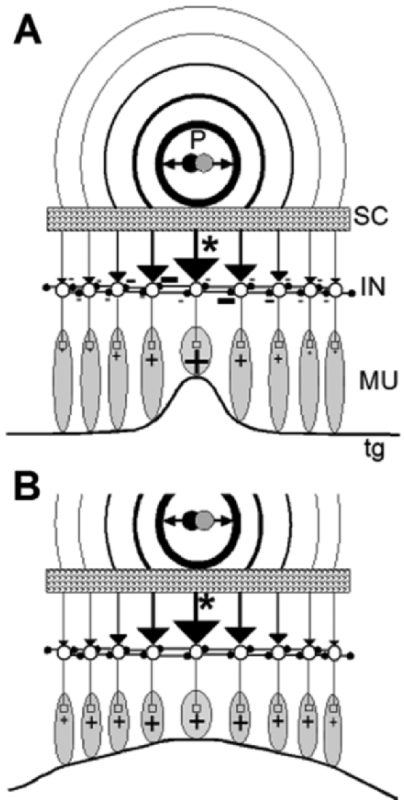

Figure 7. Model of the proposed food groove mechanosensory-motor pathway. Waves of vibration spread out from a probe (P) to hit sensory cells (SC) in the tentacular groove – the strongest response is from those sensory cells closest to the stimulus; response intensity is given by the heavy-headed arrows. Lateral inhibition between proposed interneurons (IN) of the nerve net is indicated by the open circles connected by lateral processes with (−) symbols at the filled circles indicating synapses; lateral inhibition could also occur between mechanoreceptors. The degree of inhibition is indicated by (−) symbol size. Proposed interneurons also synapse on muscle cells (MU). Neuromuscular junctions are marked with a square, and level of excitation by the size of the (+). Note that lateral inhibition will be greatest in the muscles immediately adjacent to the centralmost pathway, marked by an asterisk. 'tg', edge of the transport groove. (A) Normal motor response showing a focal eversion directly opposite the probe. (B) Mg^{2+} anesthesia showing eversion over a larger region of the transport groove.

Mechanosensors may send processes directly to transport groove muscles or synapse upon interneurons within the ectodermal nerve net. Mutually inhibitory interactions between mechanoreceptors or interneurons would be expected to result in reduced muscular activity lateral to the stimulus. Such lateral inhibition would produce a focal eversion of the transport groove near the prey.

That we can test mechanosensitivity in a reduced preparation of this ctenophore is promising for future research, and so we will use reduced preps. We will seek to identify mechanosensors by staining with the dye FM1-43, recently shown to be a fluorescent marker of large, nonselective mechanosensory channels in stereocilia (Myers et al., 2003) during eversion. We will perform high precision mechanostimulation coupled with patch clamp recording to test our hypothesis that g-cilia are mechanosensors. We will use intra- or extracellular recording to try to identify the motor pathway that triggers eversion. We will use patch or intracellular recording to seek mutual inhibition among neurons or mechanosensors parallel to the food groove axis. The embedded tentacular tract (Moss et al., 2001) will be a first target for studies of mutual inhibition underlying lateral inhibition.

Study of the *Mnemiopsis* food groove complex promises to provide insight into ctenophore sensory-motor mechanisms and pathways. Furthermore, it will aid in our understanding of the role of this ctenophore in the environment, and shed light on the phylogeny of mechanosensory systems in lower invertebrates.

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Primary inhibition: a mechanism for sudden stoppage of metachrony in ctenophores

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Key words: Comb plate, cilia, metachrony, neurociliary regulation, *Beroë cucumis*, *Pleurobrachia pileus*

Abstract

The ctenophores *Beroë cucumis* and *Pleurobrachia pileus* exhibit rapid stoppage of comb plate beating, without accompanying muscular contraction, in response to an orally applied mechanical stimulus. This phenomenon, termed 'primary inhibition' by Göthlin, was re-examined by high-speed video microscopy and intracellular recording. The most remarkable features of this event were that (1) inhibition was associated with only one or two comb plates in the row, (2) inhibition occurred nearly anywhere in the ciliary beat cycle, (3) the inhibited plate acted as a mechanical blockade to the propagation of additional metachronal waves, and (4) a single depolarizing post-synaptic potential occurred nearly simultaneously with comb plate inhibition. *B. cucumis* and *P. pileus* have evolved a neurally controlled behavior to rapidly stop comb plate metachrony.

Introduction

Comb plates are very large compound ciliary organelles that can be up to 2 mm long. Comb plate cilia arise from the apical end of a densely packed ridge of several thousand columnar epithelial cells called polster cells. Polster cells have been previously shown to be innervated, probably by the nerve net (Hernandez-Nicaise, 1974; Tamm, 1982). The polster cells can be up to 75 μm long in larger ctenophores, but are bottle-shaped and taper to a tip only a few micron across. Approximately 125 cilia arise from the apical end of each polster cell.

Locomotory comb plates are arranged along eight meridional comb rows, the activity of which are coordinated by the aborally located statocyst. The statocyst contains four compound 'balancer cilia' that together bear on their tips a group of cells that secrete the statolith (Tamm, 1982). Balancer cilia respond to differential weighting by the statolith with either an increase or decrease in the beat rate, dependent upon the animal's 'mood,' i.e. whether the animal swims with either negative (up mood) or positive (down mood) geotaxis. Balancer movement stimulates beating in a narrow ciliated tract called the ciliated groove (CG), which extends to the aboral-most comb plate of each row (Tamm, 1982). Beating of the first (i.e. aboral-most) comb plate is mechanically evoked by the CG, which broadens to form a delta-like field of cilia that touches the center of the aboral side of the first comb plate. Each wave of beating by the CG elicits a beat cycle in the first comb plate. In cydippids such as *Pleurobrachia pileus* and beroids such as *Beroë cucumis*, forward beating of plates orally positioned to the first plate is dependent solely upon hydrodynamic interactions between adjacent comb plates.

In contrast, lobate ctenophores such as *Mnemiopsis leidyi* and *Bolinopsis infundibulum* bear interplate ciliated grooves (ICGs) that span the distance between comb plates (Tamm, 1982). Coordination of beating between even distant plates requires only an intact ICG (Tamm, 1973).

Comb plate beating is therefore coordinated strictly mechanically in all species. Since the statocyst is at the aboral end of the animal, metachronal waves of beating begin at the aboral end of each comb row and progress orally. The comb plate power strokes are directed aborally; thus undisturbed, forward-swimming ctenophores swim mouth first. In *Pleurobrachia*, comb plates rest quietly unless stimulated, but can beat at up to ~20 Hz, undergo both forward and reverse beating, a multitude of transitional asymmetries between reverse and forward beating, and a nonrhythmic 'silent reorientation' (Tamm & Moss, 1985).

Although *coordination* of both forward and reversed comb plate beating is mechanical, *mode* and *orientation* of beating are regulated by ionic and neural pathways. The cydippid *Pleurobrachia* has a subectodermal network of fine neurites (Hernandez-Nicaise, 1974). *Pleurobrachia* comb plates are excitable (Moss & Tamm, 1986, 1987). Neural regulation of comb plate beating in ctenophores was demonstrated for *Pleurobrachia* (Moss & Tamm, 1993), although the precise nature of the pathway was not determined. To date, the only study of neurociliary regulation of beating to demonstrate direct recording from a pre-synaptic neuron and the polster cells and comb plate it innervates is that of Mackie et al. (1992) for *Euplokamis*.

Primary inhibition was described by Göthlin (1920) as the cessation of comb plate beating without retraction of the row into the mesogloea. In contrast, stoppage with retraction was called secondary inhibition. Göthlin inferred that primary inhibition is dependent on neural input.

This manuscript describes four new aspects of primary inhibition in *Beroë cucumis* and *Pleurobrachia pileus*. (1) Primary inhibition involves a sudden local inhibition of beating at one or two plates of a row. (2) Inhibition blocks metachrony along the entire row. (3) Single-shock electrical stimulation of reduced 'split-row' preparations evokes a single depolarizing post-synaptic potential accompanied by inhibition. (4) Inhibition appears to occur during any phase of the active beat cycle. We confirm the neural basis of primary inhibition suggested by Göthlin, and describe the mechanism of stoppage.

Materials and methods

Animals

All animals were collected by dipping to minimize physical damage to the ectoderm. Animals were held in buckets or plankton kreisels in the laboratory.

Intact animals

Intact ctenophores were immobilized by pinning in Sylgard-lined (Dow Corning, MI) Petri dishes and positioned so that the comb plates could be viewed in profile. Comb plate motor responses were recorded with a split-field video camera (model XE-S, Xybion Corporation, Sunnyvale, CA) equipped with a macro lens. Images were recorded on video tape for subsequent still-field playback.

Mechanical stimuli were manually applied to intact ctenophores by tapping the oral region with a polished glass probe. The ctenophore predators *Beroë cucumis* and *Beroë ovata*, the cydippid *Pleurobrachia pileus*, and the lobate species *Mnemiopsis leidyi* and *Mnemiopsis mccradyi* were all examined. Only *B. cucumis* and *P. pileus* displayed primary inhibition.

Intracellular recording

Intracellular recording was carried out on 'split-row' preparations of *Pleurobrachia* comb rows as described previously (Moss & Tamm, 1986). Briefly, sections of a comb row and the immediately adjacent ectoderm were microsurgically excised with very fine iridectomy scissors. The preparation was immobilized in a flow-through bath by pinning with fine cactus spines (*Opuntia* spp.) to a Sylgard wall of the chamber so that the cut edge of the polster cells was positioned up and accessible to a micro-electrode. Recording was carried out with intracellular amplifiers (model 701, World Precision Instruments) as in Moss & Tamm (1986).

Microelectrodes were pulled on a horizontal puller (Industrial Science Associates, or model p87, Sutter Instruments) to have a longitudinal resistance $\geqslant$70 MΩ, and were adjusted to be very fine, long, and flexible. Intracellular recording was feasible only with *Pleurobrachia pileus*, which has a

firm, relatively non-contractile body. It was not successful with *Beroë cucumis* because in that species muscular contractions prevented stable electrode impalement. Microelectrodes were bent in a microforge to allow them to vertically approach the cut edge of the preparation while viewing from the cut edge (for more details, see Moss & Tamm, 1986).

Electrical stimuli were applied to reduced preparations with glass suction electrodes attached to the ectodermal surface. Biphasic shocks varied from 20 to 50 V and were of 5 ms duration. The indifferent platinum electrode was exposed only at the very tip, and stimulation and recording electrodes were positioned to reduce stimulus artefacts.

Polished glass microprobes used for mechanical stimulation of reduced preparations were attached to piezo ceramic bimorph benders held in a micromanipulator. The piezo benders were forced to bend by applying single 5–20 V, 10 ms, square waves from a stimulator. This caused the probe to rapidly tap and retract from the ectoderm.

Video tape records of reduced preparations were made with a dissecting microscope equipped with a video port. A special effects generator superimposed the image of an oscilloscope, which displayed both the intracellular recording and the stimulus current, while an instrumentation recorder recorded the bioelectrical signal. Ciliary beat frequency was determined by counting the number of video fields to complete a beat cycle, converting them to time, and taking the inverse. Records of intracellular activity therefore were directly correlated with the ciliary motor response. Plots of ciliary motor activity and concurrent bioelectrical activity were generated as in Moss & Tamm (1986).

Results

Whole animals

Six *Pleurobrachia* and four *Beroë* displayed primary inhibition in response to light taps of the oral region. Primary inhibition stopped comb plate metachrony (i.e. the waves of beating). One or two comb plates typically stopped in mid-beat cycle and persisted in a semi-erect position partway through the beat cycle. The local comb plate stoppage presumably produced a physical blockade to the passage of additional metachronal waves so waves could not propagate past the inhibited plates. Figures 1 and 2 show blockade of metachrony by primary inhibition in *Beroë* and *Pleurobrachia*, respectively. Waves oral to the site of inhibition at the time of inhibition propagated to the end of the row (Fig. 1).

Primary inhibition in different animals (and in different rows) occurred anywhere along the comb row; however, in any one animal, inhibitory responses of a given row usually occurred in only one or, at most, two adjacent comb plates (Fig. 2A). One *Pleurobrachia* and one *Beroë* displayed two sites of inhibition on one row. Sometimes more than one wave was blocked at the inhibition site. In the case shown in Figure. 2B, the site of inhibition appeared to move aborally after one tap; this could be seen with the progressively more aboral inhibition of successive waves.

Closer examination of the movement of individual comb plates undergoing primary inhibition revealed that the comb plate ceased active movement at any stage during the comb plate beat cycle. Figure 3 shows three different comb plates, from three different intact *Pleurobrachia*, that display inhibition at various stages during the beat cycle. Since primary inhibition could be detected only during active beating, (i.e. during the power or recovery stroke), it was not possible to determine if inhibition could have occurred during quiescent periods between beats. Inhibition resulted in a distinct decrease in the sharpness of curvature of the comb plate (Fig. 3), suggesting that it affected the entire length of the comb plate cilia.

Reduced preparations

Primary inhibition was elicited in split-row preparations by the application of single taps to the ectodermal surface with a miniature glass probe attached to an electrically driven piezoceramic bimorph. Single biphasic shocks applied to the ectodermal surface also evoked inhibition. Ten preparations were stimulated with fast trains of shocks as described previously to induce fast forward beating (Moss & Tamm, 1986). Single mechanical or electrical stimuli were then applied during rapid continuous beating. As with

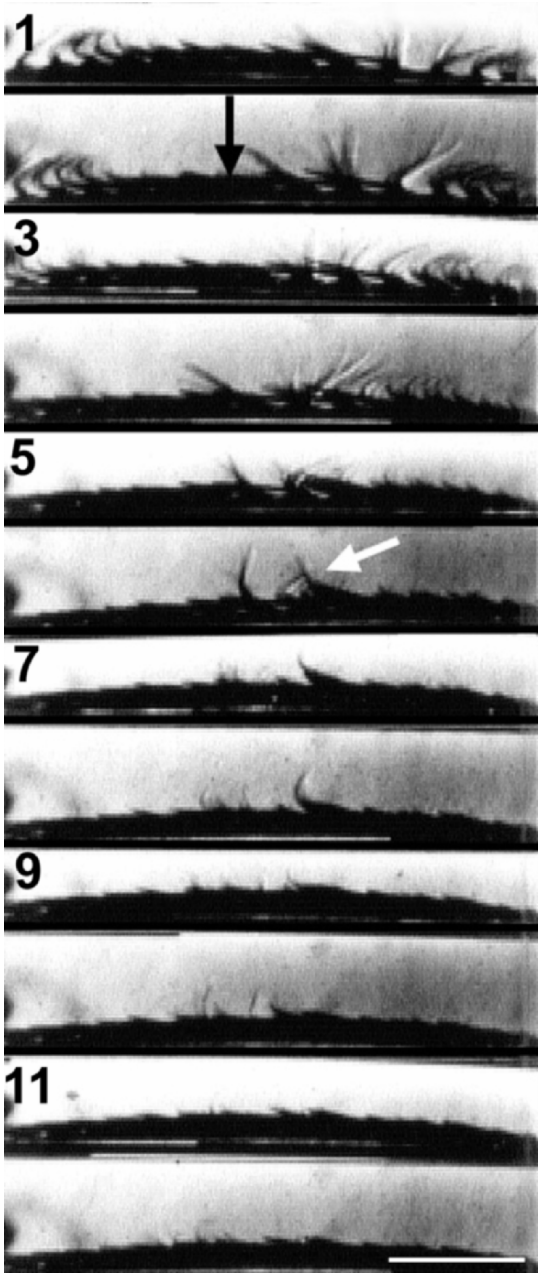

Figure 1. Primary inhibition in *Beroë cucumis*: a single comb row in profile, through time. Subfield images from a splitfield video camera. Three subfields/full field; time between subfields ~5.5 ms. Mouth is to the left. Mechanical stimulus was applied near lips between subfields 1 and 2. Metachronal waves travel from the right toward the mouth in images 1–4, but die out with the two comb plates indicated by black and white arrows. The black arrow indicates the oral-most comb plate to beat actively; it then undergoes primary inhibition. The white arrow indicates a comb plate that executes a full beat but then undergoes primary inhibition, and blocks any further metachrony. Scale 1 mm.

the intact animal, inhibition occurred at only a few comb plates.

Intracellular recording

Intracellular recordings were made from polster cells of comb plates displaying inhibition. In such preparations, a depolarization occurred immediately prior to and during inhibition of beating. Inhibition occurred within the resolution of the video recording system, i.e. the inhibition began within one video field (16.7 ms) of the onset of the stimulus. Transmission of the signal was approximately 110 mm/s ($n = 3$) as measured from electrically stimulated preparations (preparations were very small, usually <20 mm overall, so the conduction rate is only approximate). The comb plate resumed beating within 1 s after decay of the single depolarization (Fig. 4).

The depolarizing response to single shocks had an average amplitude of 24.5 ± 2.5 mV and decayed back to the resting potential in less than 1 s (0.6 s ± 0.03, $n = 12$). The depolarization to mechanical stimuli measured 19.5 ± 1.1 mV and lasted for 0.58 ± 0.125 s ($n = 10$). The similarity of the responses suggest that the mechanical probe and single electrical shocks stimulated the same conducting system. Sometimes spike-like responses accompanied the electrical-shock induced depolarization (Fig. 4C) although this was a rare occurrence. If electrical stimuli were given with the suction electrode off-prep but in the recording bath, no response was seen. Thus, the response was not to a field effect from the stimulating electrode.

Discussion

Neuronal pathways in ctenophores

Epithelial innervation was reported by Horridge & Mackay (1964) and Horridge (1966) via observations of ctenophore tissues by TEM. Hernandez–Nicaise established not only the overall organization of the *Beroë* and *Pleurobrachia* nerve net, but also the pre-synaptic triad anatomy of ctenophore synapses (1973, 1974), a neuronal criterion structure widely used to this day (e.g. Tamm & Tamm, 1981, 1995, 2002; Tamm, 1982; Mackie et al.,

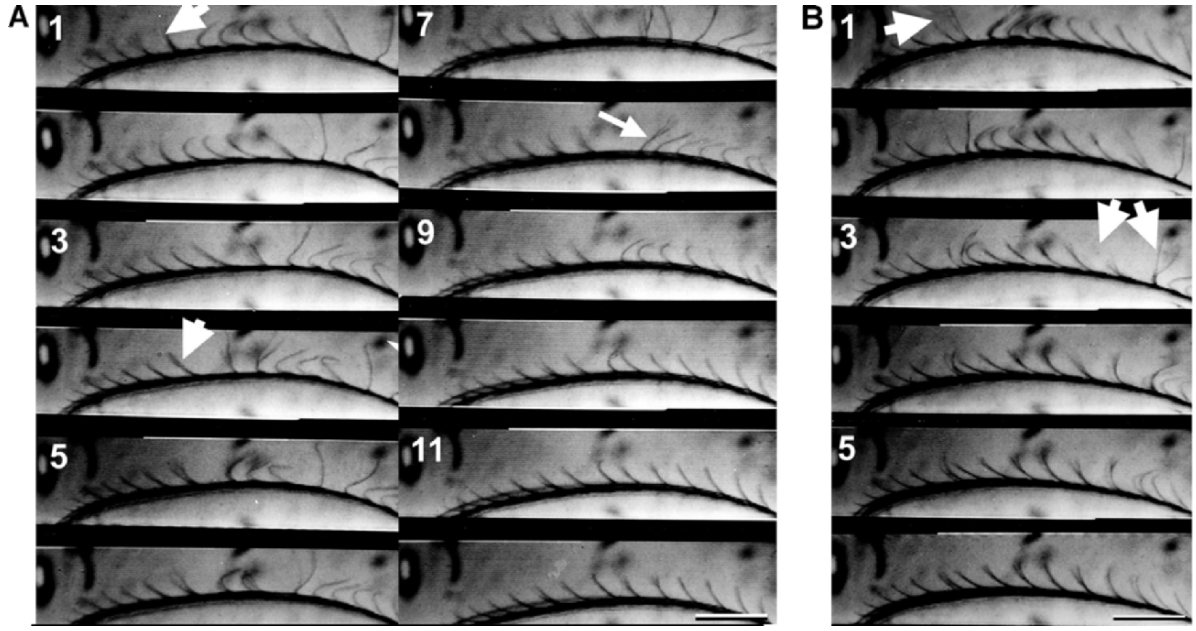

Figure 2. Primary inhibition in *Pleurobrachia pileus*. Orientation as in Figure 1. (A) Two metachronal waves are shown. Both are shut down by the comb plate indicated by arrows in subfields 1 and 4, then by the plate indicated in subfield 8. (B) A second example, showing oral-to-aboral spread of inhibition. One tap caused primary inhibition in the comb plate indicated by the arrow in subfield 1. A subsequent wave began at the aboral end but died out between the two comb plates indicated by the two arrows in subfield 3; note that the site of inhibition is about 1 mm aboral to the first site. Scales 400 μm.

1992). Intracellular recording from *Pleurobrachia* polster cells showed that ciliary comb plate reversal occurred in response to depolarizing post-synaptic potentials in the polster cells (Moss & Tamm, 1986). Depolarization of the polster cell bodies evokes action potentials along the length of comb plate cilia, but not the cell bodies, which themselves appear to be inexcitable (Moss & Tamm, 1987). During a bout of reversal, steady-state depolarization was identical across the polster ridge, while individual cells and cilia varied with regard to post-synaptic and regenerative activity (Moss & Tamm, 1986).

Extracellular recording from intact *Pleurobrachia* (Moss & Tamm, 1993) revealed that electrical stimulation of a tentacle would drive spikes in a one-to-one manner in the tentacle. Volleys of tentacular action potentials appeared to evoke slow phasic activity in the tentacular bulb, which in turn drove phasic trains of action potentials in the comb plates of that hemisphere, resulting in calcium spiking in the comb plates and reversal of the power stroke. Small depolarizing post-synaptic potentials, timed one-for-one with each electrical stimulus applied to the tentacle and indicative of a through-conducting pathway, were also seen in polster cells. Thus, it is not surprising to find neurally regulated comb plate inhibition in *Pleurobrachia*. Similar work on *Beroë* is much more difficult to perform; efforts to date have not been successful because the animal is very muscular and responds to electrical shock stimuli with vigorous muscular contractions.

Rapid stoppage of metachrony

The single most important result from this study is that primary inhibition rapidly blocks metachrony by producing a physical blockade with 1 to 2 plates. Thus, propulsive activity of the comb row is blocked during the timing of 1–2 metachronal waves. Ctenophores swim efficiently because all of the comb plates of one row generate jets of water, a result of the collective beating activity of that row (Barlow et al., 1993). Jetting activity requires continuous comb plate beating. Thus, quick interruption of metachrony is very effective at shutting down forward motion of freely swimming animals.

Primary inhibition appears to result in the loss of comb plate mechanosensitivity, because while undergoing inhibition the comb plate is unresponsive to the mechanical activity of aboral plates. Instead, the inhibited plate extends with a low degree of curvature away from the body, where it acts as a wall to block mechanical coordination.

Focal inhibition

During primary inhibition, localized inhibition of 1–2 comb plates parsimoniously shuts down beating of nearly all of the plates in a row. Plates undergoing stoppage experience a brief depolarization, while surrounding comb plates may be unaffected. If so, there could be distinct foci of innervation that serve this function. Primary inhibition probably uses a different neural pathway from the one used for unilateral reversal during feeding. This is because during prey capture, all subtentacular comb plates and 50% of the subsagittal plates on the prey-catching side of the animal display reversal (Tamm & Moss, 1985). In addition, Moss & Tamm (1986) showed that for reversal to occur, there must be trains of calcium-dependent action potentials in all comb plates undergoing reversal (Moss & Tamm, 1987). The trains of action potentials are produced by efferent output from the tentacular bulb, which acts as an integrating center for signals from the tentacle. The action potentials occur in comb plates throughout the hemisphere of the stimulated tentacle (Moss & Tamm, 1993). Thus, the comb plates seem to share innervation that is involved in the unilateral reversal associated with feeding, while primary inhibition involves only one or two plates of a row.

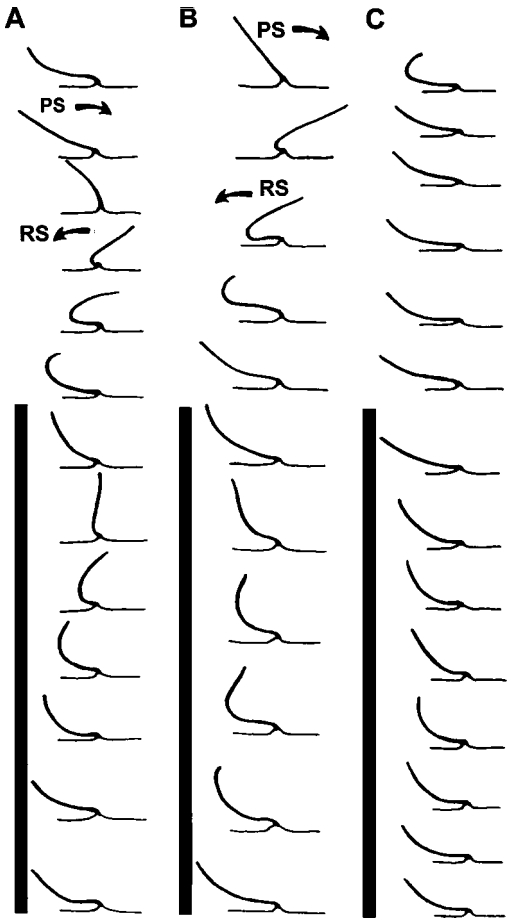

Figure 3. Comb plate motor responses during inhibition. Three examples traced from video images of intact *Pleurobrachia*. (A) Comb plate inhibition occurs midway during the second power stroke. (B) Inhibition occurs early in the power stroke. (C) Inhibition occurs at the beginning of the power stroke. Heavy vertical bars indicate the period of primary inhibition. PS – power stroke. RS – recovery stroke.

The primary inhibition pathway might use the previously mentioned through-conducting pathway, or yet another entirely unknown pathway (in either case it must produce focal responses – highly unlikely in the through-conducting pathway). That the pathway is ectodermal is apparent from the functionality of the split-row preparations, which include only the ectoderm and a thin layer of the mesogloea and outer part of the canal that underlies the comb row.

Adaptive value

Primary inhibition is probably a protective adaptation for the coastal ctenophores *Beroë cucumis* and *Pleurobrachia pileus*. It could serve as part of an avoidance mechanism. Coastal ctenophores often encounter obstacles such as the air/water interface, vegetation, and other animals (including fish). When they encounter such obstacles, they stop swimming, and reorient to swim in another direction (Moss, unpublished observation). Primary inhibition stops forward swimming, and so could have survival value, particularly if the obstacle encountered is a be-

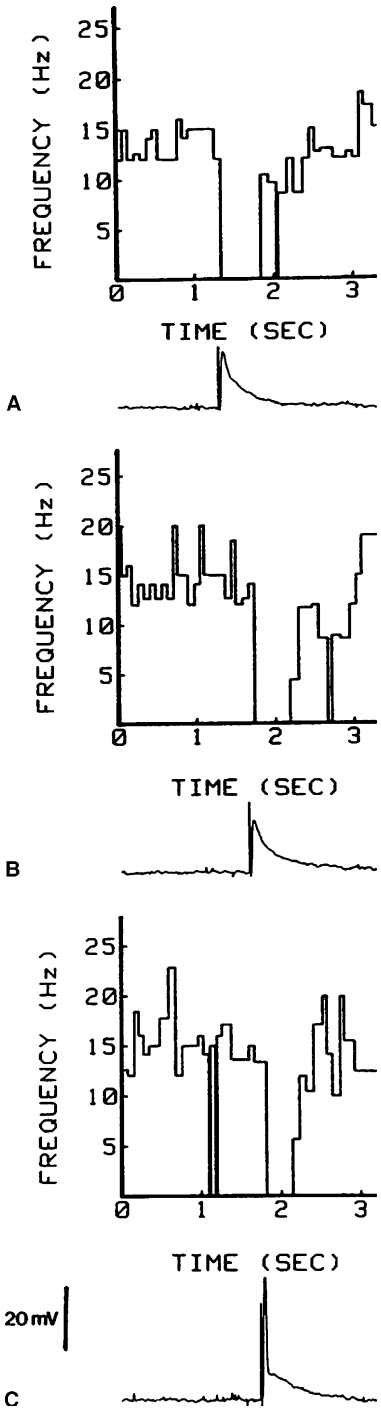

Figure 4. Polster cell electrical response and temporary cessation of comb plate beating. Three examples are shown in 4A–C. The graphs indicate beat frequency and direction as a function of time. 5 ms, 50 V, single shock biphasic stimuli were applied after stimulating rapid continuous beating with a 10 s train of 5 ms, 5 V, 10 s^{-1} stimuli.

roid, which preferentially preys on ctenophores – even *Beroë cucumis*, because that species displays cannibalism. Primary inhibition also shuts down the colorful shimmering of the comb rows, which might attract fish, many of which prey on ctenophores (Purcell, 1985; Cowan & Houde, 1993; Purcell & Arai, 2001).

Primary inhibition was observed only in *Beroë cucumis* and *Pleurobrachia pileus*. Despite extensive testing, *Mnemiopsis leidyi*, *M. mccradyi*, and *B. ovata*, all of which are coastal ctenophores, failed to display it. It is unclear why some species have primary inhibition while others lack it.

Mechanisms of primary inhibition

Primary inhibition is remarkable in that it is an exceptionally sudden stoppage. Inhibition can occur during mid-power stroke and may occur during any stage of the beat cycle. This implies that coordinated activity of the motility enzymes (dynein, radial spokes, etc.) are simultaneously (or at least very rapidly and with little lag) modified all along the length of the cilium. We know that comb plate beat rate and direction are modulated by intraciliary calcium concentration (Nakamura & Tamm, 1985) and that the type of regulation is probably dependent upon the spacial distribution of calcium influx (Tamm, 1994). Clarification of mechanochemical coupling during comb plate primary inhibition will require further study. That such studies can be even considered is testimony to the value of ctenophores as model systems for the understanding of basic ciliary processes.

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Part III
Cnidae

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Nematocysts of *Polypodium hydriforme*, a cnidarian parasite of acipenseriform fishes

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Key words: Polypodium hydriforme, nematocyst, atrichous isorhiza, holotrichous isorhiza, penetrants, symmetry

Abstract

Structure of nematocysts from *Polypodium hydriforme* was studied by scanning and transmission electron microscopy. Two nematocyst types were observed: holotrichous isorhizas with minute spines, and true atrichous isorhizas. Among holotrichous isorhizas four morphological categories were distinguished based on differences in morphology of their discharged stinging tubules. The cnidom of *P. hydriforme* possesses no specialized penetrant types. Instead, *Polypodium* uses holotrichous isorhizas as penetrants during prey capture. Stinging tubules of holotrichous isorhizas display two helical rows of tiny spines in the discharged state and possess two helical folds in the undischarged state, thus corresponding to double-helical symmetry. Double-helical symmetry has never been described for nematocysts of any cnidarians, thus being a peculiar feature of *P. hydriforme*.

Introduction

Polypodium hydriforme Ussov is an aberrant cnidarian parasite, possessing so many unique features that it has been placed into a separate class, Polypodiozoa Raikova 1988 (Raikova, 1988, 1994), under a superclass Hydrozoa (Bouillon & Boero, 2000). During the parasitic phase of its life cycle, *P. hydriforme* develops intracellularly inside oocytes of acipenseriform fishes. When fishes spawn, the parasite hatches into the water and fragments into free-living specimens.

Free-living *P. hydriforme* is a fresh-water animal of approximately the same size as *Hydra*, but quite different in its body structure (Fig. 1A). It has no stalk or peduncle; instead it uses four specialized 'walking tentacles' for attachment and locomotion. For catching prey, *P. hydriforme* uses eight long and slender 'sensory tentacles.' *Polypodium hydriforme* has a mouth cone always pointing upward. Free-living *P. hydriforme* specimens possess biradial symmetry and continually reproduce by means of longitudinal fission, starting at the aboral pole.

Cnidocytes (stinging cells) are used by free-living *P. hydriforme* for prey capture (Lipin, 1911), for tentacle adhesion during locomotion (Lipin, 1911), and for gametophore attachment to host fishes (Smolyanov & Raikova, 1961). The structure and development of cnidocytes in *P. hydriforme* was studied with both light (Lipin, 1911; Raikova, 1960, 1961) and electron (Raikova, 1978, 1990) microscopes. The cnidocytes of *P. hydriforme* have a cnidocil lacking rootlets, with its kinetosome localized directly on the nematocyst lid (Raikova, 1990). This feature has never been observed in cnidocytes of other cnidarians. Preliminary data on the structure of discharged nematocysts were published in Russian (Ibragimov, 1999, 2002); in the present paper they are revised and supplemented.

Material and methods

The material was collected in the Volga River. Parasitic stolons of *P. hydriforme* were isolated

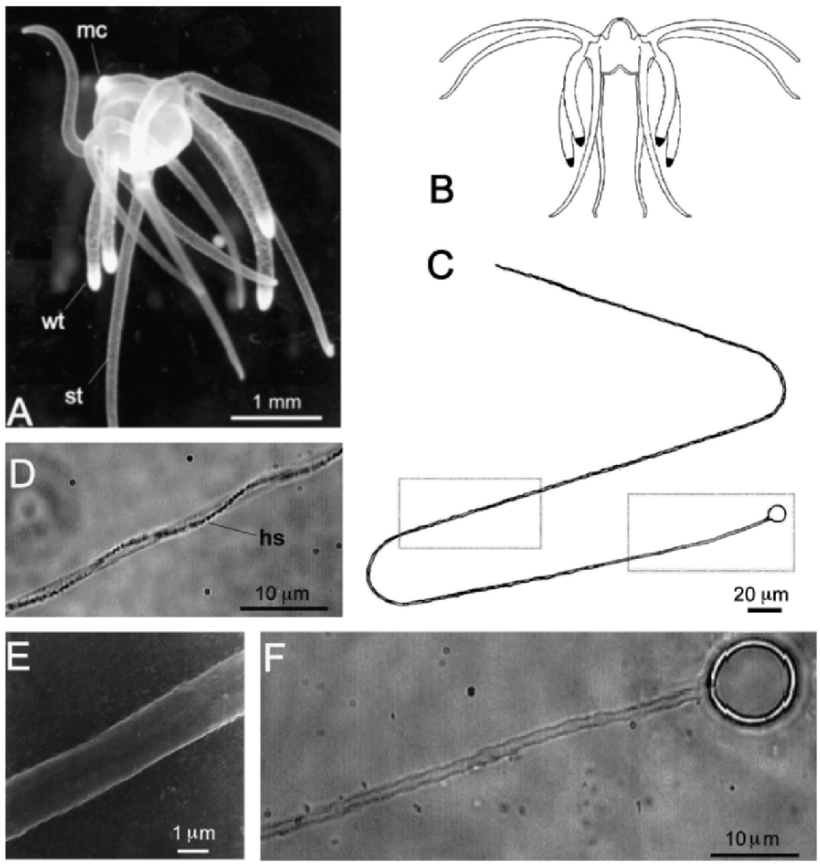

Figure 1. *Polypodium hydriforme* and atrichous isorhizas. (A) LM of a free-living specimen. (B) Distribution of atrichous isorhizas (marked black on the tips of the tentacles) in free-living *P. hydriforme*. (C) Discharged nematocyst. (D) LM of a discharged stinging tubule (central region) with a helical glutinous string. (E) SEM of a discharged stinging tubule (central region). (F) LM of a discharged nematocyst and proximal region of a stinging tubule. Abbreviations: hs, helical glutinous string; mc, mouth cone; st, 'sensory tentacle'; wt, 'walking tentacle.'

from infected prespawning eggs of sterlet (*Acipenser ruthenus*). Free-living specimens of *P. hydriforme* were reared from these stolons and cultivated in plastic dishes filled with fresh water. Specimens were fed with *Tubifex* worms once a day.

To obtain nematocyst discharge, two methods were used:

(1) A drop of diluted ethanol was added to a specimen under a cover slip. In the course of time a sufficient number of nematocysts discharged. The advantage of this gentle method was that it resulted in the normal discharge of the nematocysts, and the shape of their tubules remained intact. The disadvantage of this method was that certain categories of nematocysts discharged more readily than others.

(2) A specimen was placed into 0.1% SDS (sodium dodecyl sulfate) solution. In less than 20 min the specimen became dissolved, with most of its nematocysts discharged. The disadvantage of this method was the abnormal discharge observed in some nematocysts: the distal part of their stinging tubule failed to complete its eversion.

For transmission electron microscopy, a drop of SDS-treated nematocyst suspension was placed on formvar-coated grids and air-dried. Unstained grids were then examined using a JEM-100C or JEM-7A transmission electron microscope.

To study undischarged nematocysts, whole *P. hydriforme* specimens were fixed and processed for electron microscopy (for details see Raikova,

1990). To study nematocyst impact on prey objects, we have removed *Tubifex* worms from the tentacles and mouth of *P. hydriforme* specimens and then processed them for transmission electron microscopy in the same way.

For scanning electron microscopy, specimens were treated according to Raikova (1995). Occasionally discharged nematocyst tubules were examined using a Hitachi S-570 or Leo S 420 (Oberkochen) scanning electron microscope.

Results

Nematocysts of *P. hydriforme* fall into two types of classification by Weill (1934): atrichous isorhiza and holotrichous isorhiza.

Atrichous isorhiza

Cnidocytes with atrichous isorhizas are localized at the tips of walking tentacles only (Fig. 1B, C). We confirm that they are used for tentacle adhesion during locomotion (Lipin, 1911). No spines or folds can be distinguished on their discharged tubules (Fig. 1E). Instead, stinging tubules of atrichous isorhizas usually bear a single helical string of glutinous substance twined round them in irregular coils (Fig. 1D). Only proximal sections of stinging tubules lack the helical glutinous string (Fig. 1F).

Holotrichous isorhiza

Among holotrichous isorhizas, four categories can be distinguished based on morphology of their discharged tubules. Each of these four categories has its own pattern of distribution and presumably a unique function. Nematocysts with slightly twisted tubules (Fig. 2A, E) are distributed throughout the body of *P. hydriforme* except at the tips of tentacles. We suppose they have a defensive function. Nematocysts with exceptionally slender and flexible tubules (Fig. 2B, F) are localized in proximal parts of tentacles and in the mouth cone. Their function is unknown. The smallest nematocysts, with their tubules slightly tapering toward the distal end (Fig. 2C, G) are localized in sensory tentacles and in the mouth cone. They are especially numerous and function as penetrants during prey capture (Fig. 3A). Nematocysts of the last category resemble previous nematocysts, but their tubules have a narrow portion close to the capsules (Fig. 2D, H). In addition they are larger, bear larger spines, are far less numerous, and are localized only at the tips of sensory tentacles. Based on their similar morphology, we suppose these nematocysts could also function as penetrants, but we have never seen them being discharged into prey.

Holotrichous isorhizas of *P. hydriforme* possess two helical rows of minute spines on their tubules (Fig. 2I). These spines are up to 0.1 μm high, have no signs of mineralization, and look like protrusions of the nematocyst tubule wall (Fig. 2J, K). Two helical fissures separate the two rows of spines (Fig. 2L), and multiple lesser transversal fissures separate adjacent spines in each row (Fig. 2L). The rows of spines continue throughout the length of the tubules to the distal end, and the spines are of nearly the same size. Therefore we defined these nematocysts as holotrichous isorhizas.

Nematocysts and prey capture

Tubifex worms and *Paramecium* ciliates were used to study the impact of nematocysts on prey objects. In both cases the same category of holotrichous isorhiza nematocysts was observed to be discharged (Fig. 2C, G).

In nematocysts discharged into prey, a proximal part of their tubules about 5–20 μm long remain visible (Fig. 3A, B) above the surface. The rest of the tubule is in the prey (Fig. 3C). Often the nematocyst does not complete its discharge and a distal part of the stinging tubule remains inverted and folded inside a discharged proximal part (Fig. 3D).

Undischarged nematocysts

Undischarged stinging tubules of atrichous isorhizas are tightly folded inside their capsules, showing irregular profiles at TEM sections (Fig. 3E). In undischarged holotrichous isorhizas, stinging tubules are packed more loosely, and in TEM sections S-like profiles (Fig. 3E) of nematocyst tubules can be recognized.

Discussion

All nematocysts of *P. hydriforme* were previously identified as atrichous isorhizas (Weill, 1934)

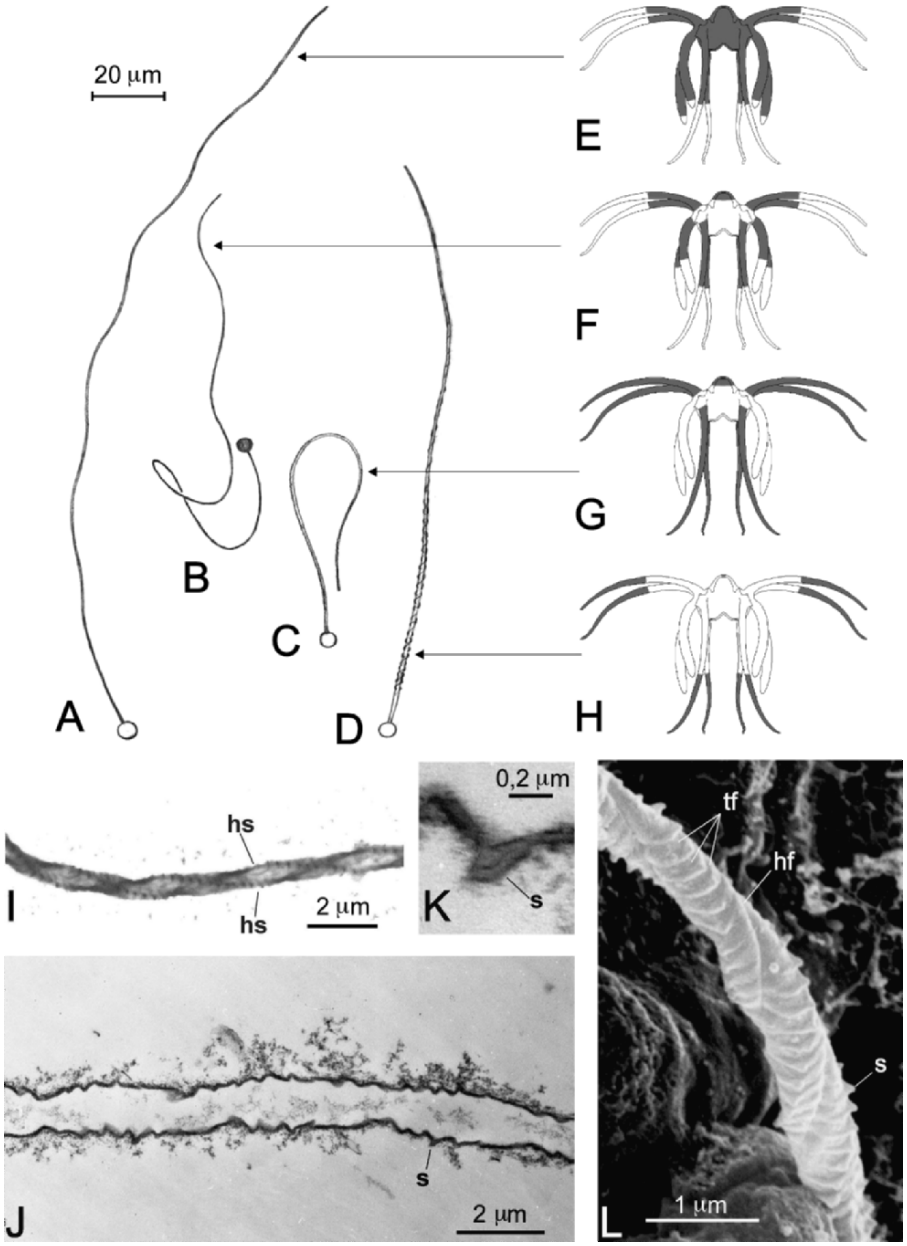

Figure 2. Holotrichous isorhizas from *P. hydriforme* (A, B, C, D). Discharged holotrichous isorhiza nematocysts of different categories. (E, F, G, H) Localization of the corresponding nematocysts (marked in gray) in free-living *P. hydriforme*. I. TEM of a part of a discharged stinging tubule. J. TEM longitudinal section through a discharged stinging tubule. K. Magnified part from the same section, showing a spine. L. SEM of a discharged stinging tubule. Abbreviations: hf, helical fissure; hs, helical row of spines; s, spine; tf, transversal fissure.

because no spines could have been distinguished with light microscope (Lipin, 1911; Raikova, 1960, 1961). Our study shows a presence of two nematocyst types: a) true atrichous isorhiza having no spines, but with a single helical string of glutinous material, and b) holotrichous isorhizas with two helical rows of tiny spines.

In most cnidarians there are special types of nematocysts with a shaft (such as stenoteles, euryteles, mastigophores, etc.) used in prey capture

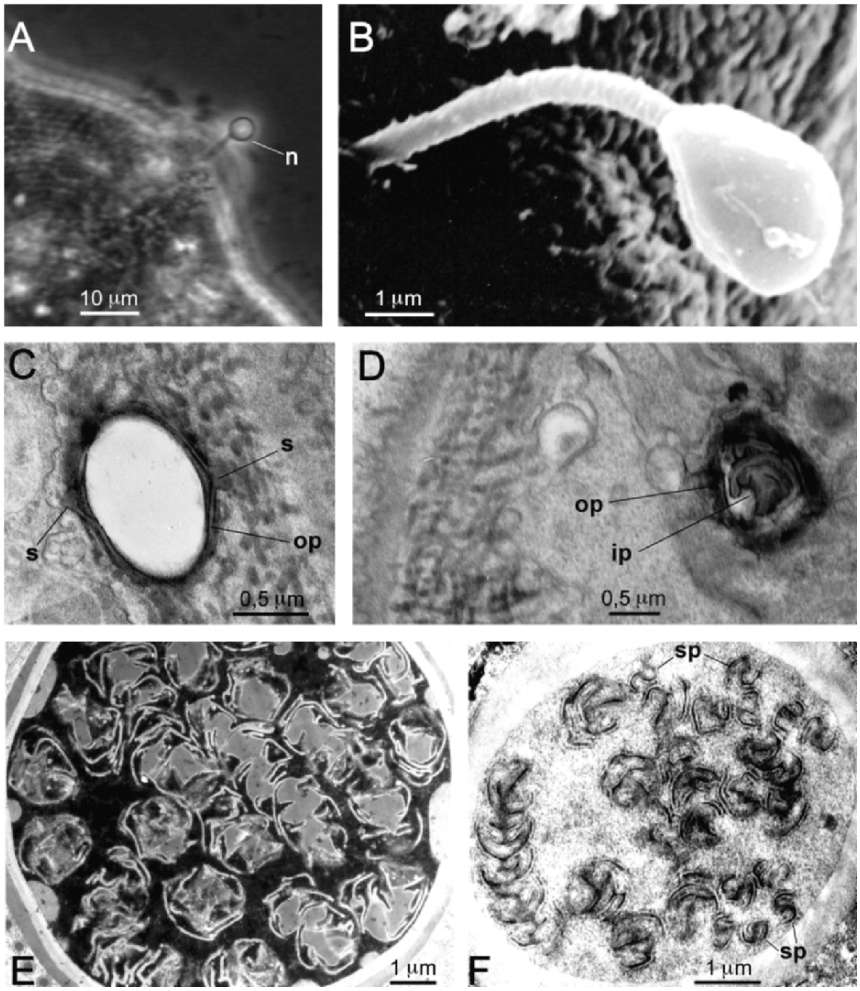

Figure 3. Nematocysts used for prey capture and undischarged nematocysts. (A) LM of a holotrichous isorhiza nematocyst, discharged into a ciliate *Paramecium* sp. (B) SEM of a holotrichous isorhiza nematocyst, discharged into a piece of debris. (C, D) TEM transversal section through a holotrichous isorhiza nematocyst stinging tubule, discharged into a *Tubifex* sp. worm. (E) Undischarged atrichous isorhiza nematocyst. (F) Undischarged holotrichous isorhiza nematocyst. Abbreviations: ip, inner part of a stinging tubule; n, nematocyst; op, outer part of a stinging tubule; s, spine; sp, S-like profiles.

to penetrate the skin of a target. However, *P. hydriforme* has no nematocysts with a shaft and uses its holotrichous isorhiza nematocysts as penetrants. Because the holotrichous isorhiza is supposed to be a most primitive nematocyst type (Bozhenova et al., 1988), we consider their use in prey capture by *P. hydriforme* as an archaic feature.

Nematocyst spines are usually arranged in three helical rows twined around nematocyst tubule (Chapman & Tilney, 1959; Picken & Skaer, 1966; Bozhenova, 1988; Lotan et al., 1996; Östman, 1999, 2000). During cnidogenesis the spines develop only after initial tubule inversion into the capsule (Watson, 1988), and so the three helical rows of spines probably correspond to three helical folds of undischarged nematocyst tubule. Both the folds and the spines correspond to triple-helical symmetry. Apotrichous isorhiza nematocysts of Narcomedusae were the only known exception, because their discharged tubules are decorated with five helical rows of spines, and undischarged tubules show profiles with five folds on transversal TEM sections (Carré et al., 1989). Holotrichous isorhiza nematocysts of

P. hydriforme described in the present paper are the second exception, because their tubules possess two helical rows of spines, and profiles with two folds in TEM sections, thus corresponding to double-helical symmetry. This fact is especially interesting, because in many species of Myxosporidia inverted polar tubules in immature polar capsules display the same double-folded pattern on TEM sections (Lom & de Puytorac, 1965; Lom & Vavra, 1965: pl. 11; Desser & Paterson, 1978; Desser et al., 1983, Canning et al., 1999: Fig. 7). Therefore immature polar tubules in some species of Myxosporidia and stinging tubules of *P. hydriforme* have the same type of symmetry, thus supporting the theory of homology between these organelles and of kinship between Myxosporidia and Cnidaria.

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Nematocyst composition of the cubomedusan *Chiropsalmus quadrigatus* changes with growth

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Key words: cnidae, ontogeny, a cubomedusa

Abstract

The nematocysts of *Chiropsalmus quadrigatus* (Cubozoa; Cubomedusa; Chirodropidae) were examined to determine if their composition changes with an increase in body size. Fixed tentacles of specimens collected in Okinawa, Japan, were homogenized and their nematocysts were observed under a differential interference contrast microscope. Six nematocyst types were observed in medusae of all sizes: microbasic mastigophores (MM), large and small trirhopaloids (lTR and sTR), holotrichous isorhizas (HI), ellipsoidal isorhizas (eI), and ovoid isorhizas (oI). Two other nematocysts, large ovoid isorhizas (loI) and microbasic euryteles (ME), were observed only in small individuals. There was also marked difference in proportion of tentacular nematocysts between small and large individuals. HI was the dominant type in small specimens, while MM and eI were predominant in large specimens. Nematocyst composition in the bell and pedalia also differed between small and large individuals. Bells of small medusae contained oI and sTR, while only oI were observed in most large individuals. The pedalia of small medusae had clusters of MM, ME, sTR, and oI. Such single clusters on pedalium bases were characteristic of small individuals. The pedalia of large individuals contained scattered oI. Tentacles of medusae are used for prey capture, so the changes in the major type of nematocysts in tentacles may reflect changes in prey type.

Introduction

Nematocyst composition of a cnidarian species may vary depending on developmental stage, life cycle, and physiological conditions (e.g. Fautin, 1988). For example, nematocyst composition differs between polyp and medusa in some scyphozoans and cubozoans (e.g. Studebaker, 1972; Arneson, 1976; Calder, 1983).

There has been little study of possible changes in the nematocyst composition due to growth within a given phase of the life cycle. Nematocyst composition in tentacles of one cubomedusa, *Chironex fleckeri*, is reported to change as medusae grow, while no such changes were observed in another cubomedusa, *Chiropsalmus* sp. (Carrette et al., 2002).

The Okinawan cubomedusa *Chiropsalmus quadrigatus* (Habu-kurage in Japanese) is an important jellyfish from a viewpoint of public health because of its stinging tentacles. However, nematocyst composition of *C. quadrigatus* has not been studied. We examined the nematocysts of *C. quadrigatus* to determine if their composition changes with an increase in body size of the medusae. This study showed that two types of nematocysts were present only in small medusae and that relative abundance of major type of nematocysts changed as the medusae grew.

Materials and methods

Chiropsalmus quadrigatus medusae were collected at ports in Okinawa, Japan, using a light. The medusae were brought alive to the laboratory and

their bell heights were measured. Parts of the medusae were fixed in 5% formalin in seawater and some of the excised tentacles were frozen. For nematocyst identification, squashes prepared from fresh or fixed tissues and nematocyst suspensions prepared from frozen tentacles were examined under a differential interference contrast (DIC) microscope (Nikon Optiphot). Nematocysts were identified according to Calder & Peters (1975), Rifkin & Endean (1983), and Williamson et al. (1996). For determination of relative abundance of each type of nematocyst in tentacles, fixed tentacles were ground using a glass homogenizer, and at least 200 nematocysts were counted.

A total of 83 individuals was used in this study. Nematocysts of tentacles, pedalia, bell, manubrium, and gastric filaments of the medusae were examined (Fig. 1), although the medusae were not always examined for all of their body parts. For example, only tentacular nematocysts were observed in some medusae.

Results

Nematocysts of Chiropsalmus quadrigatus

Chiropsalmus quadrigatus contained eight types of nematocysts: microbasic mastigophores (MM), microbasic euryteles (ME), large and small trirhopaloids (lTR and sTR), holotrichous isorhizas (HI), ellipsoidal isorhizas (eI), ovoid isorhizas (oI), and large ovoid isorhizas (loI) (Fig. 2). The last three varieties of isorhizas, eI, oI and loI, were tentatively identified according to Calder & Peters (1975). Two types of nematocysts, loI and ME, were observed only in small medusae (<ca. 10 mm bell height) and the other six types were observed in medusae of all sizes.

Nematocysts in various parts of the medusae (Table 1)

All the nematocyst types except ME were observed in tentacles of *Chiropsalmus quadrigatus*. MM, lTR, sTR, HI, eI, and oI were observed in tentacles of medusae of all sizes (3–90 mm bell height). loI were observed only in small individuals (<ca. 10 mm bell height). Tentacles from 65 individuals were used for nematocyst identification and/or determination of proportion of each nematocyst type. Relative abundance of each nematocyst type is described below.

Warts of the bells, characteristic of small individuals (<several millimeters bell height), contained both sTR and oI or only oI. At least 10 of 12 small individuals (3–10 mm bell height) examined had sTR on their bells; on the bells of large individuals (11–90 mm bell height, seven individuals), scattered oI and no sTR were observed.

A cluster of nematocysts on pedalium bases containing mainly MM was characteristic of small individuals (3–15 mm bell height, 32 individuals); half or more of these small individuals contained

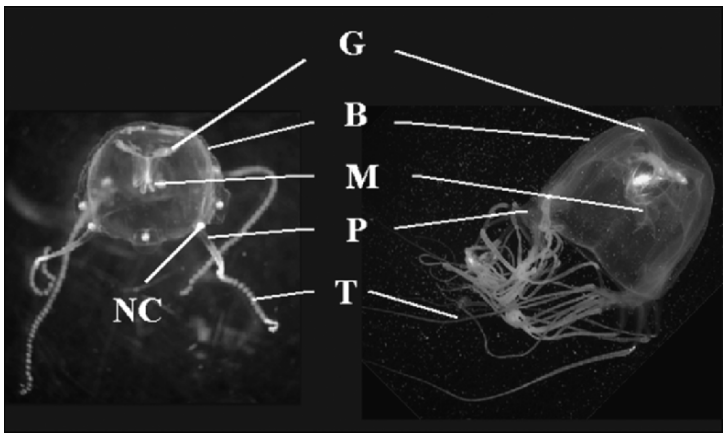

Figure 1. Photomicrographs of *Chiropsalmus quadrigatus* medusae showing various body parts. G, gastric filaments; B, bell; M, manubrium; P, pedalium; T, tentacle; NC, a nematocyst cluster on a pedalium base. Left, small medusa with bell height less than 5 mm. Right, large medusa with estimated bell height more than 50 mm.

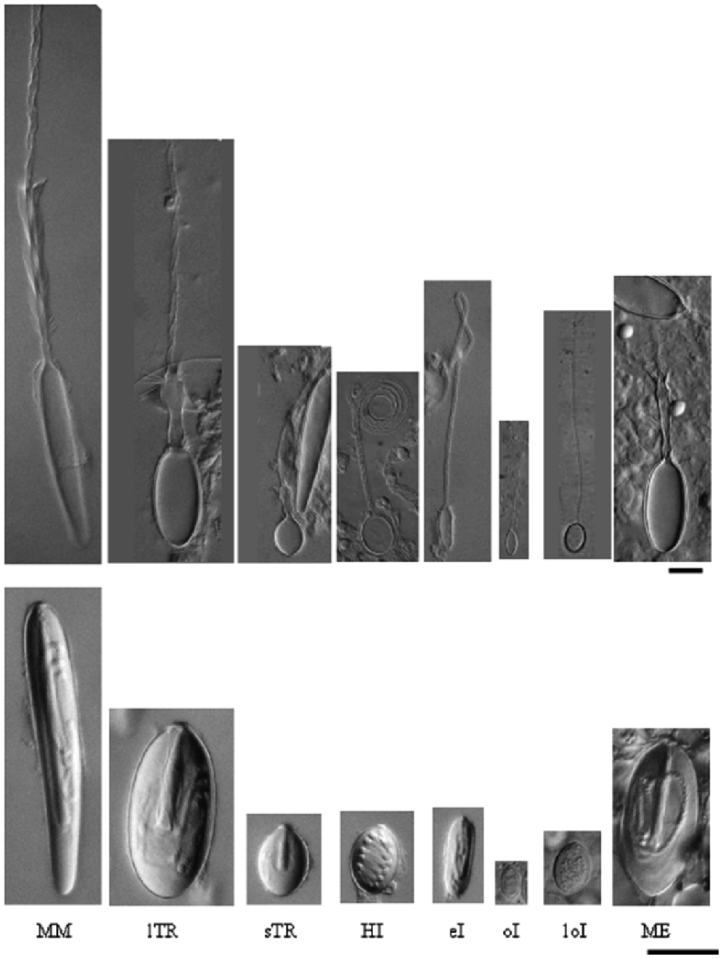

Figure 2. Nematocysts of the medusae of *Chiropsalmus quadrigatus*. MM, microbasic mastigophore; lTR, large trirhopaloid; sTR, small trirhopaloid; HI, holotrichous isorhiza; eI, ellipsoidal isorhiza; oI, ovoid isorhiza; loI, large ovoid isorhiza; ME, microbasic eurytele; scale bars = 10μm. Top, discharged nematocysts; bottom, undischarged nematocysts.

ME, sTR, and/or oI in addition to MM. The pedalia of large individuals (25–90 mm height, 13 individuals) had scattered oI; seven of these large individuals (25–33 mm bell height) had remnants of the nematocyst clusters containing MM and oI, and occasionally ME and sTR too.

Gastric filaments and manubrium of medusae 3–90 mm bell height contained sTR (12 and 16 individuals were examined for gastric filaments and manubrium, respectively).

Relative abundance of each type of tentacular nematocyst

Figure 3 shows relative abundance of each type of tentacular nematocyst plotted against bell height (31 medusae). There was a marked difference in relative abundance (%) of tentacular nematocysts between large and small individuals. HI was the dominant type in small individuals (3–15 mm bell height). The proportion of HI decreased with increasing size of medusae. The proportion of eI increased with increasing size of medusae from 3 to 44 mm bell height. eI and HI were the predominant types in middle-sized individuals (25–60 mm bell height). MM increased in proportion starting at about 40 mm bell height. Both MM and eI were predominant in large individuals (>70 mm bell height). loI decreased in proportion (from 20 to 0%) with size of medusae from 3 to 10 mm bell height.

Table 1. Nematocysts in various parts of the medusae of *Chiropsalmus quadrigatus*

Body parts	Bell height (mm)	MM	lTR	sTR	HI	eI	oI	loI	ME
Tentacles	3–10	+	+	+	+	+	+	+	–
	11–90	+	+	+	+	+	+	–	–
Pedalia	3–33	+[a]	–	+[a]	–	–	+	–	+[a]
	38–90	–	–	–	–	–	+	–	–
Bell	3–10	–	–	+	–	–	+	–	–
	11–90	–	–	–	–	–	+	–	–
Manubrium	3–90	–	–	+	–	–	–	–	–
Gastric filaments	3–90	–	–	+	–	–	–	–	–

+: present. – : few or not observed.
[a] These nematocysts observed only in the clusters of nematocyst or the remnants of the clusters at the base of pedalia.

Discussion

Nematocyst complement of *Chiropsalmus quadrigatus* was not identical but was similar to that of other chirodropids, *Chironex fleckeri* (see Rifkin & Endean, 1983; Williamson et al., 1996), *Chiropsalmus quadrumanus* (see Calder & Peters, 1975), and *Chiropsalmus* sp. (Williamson et al., 1996;

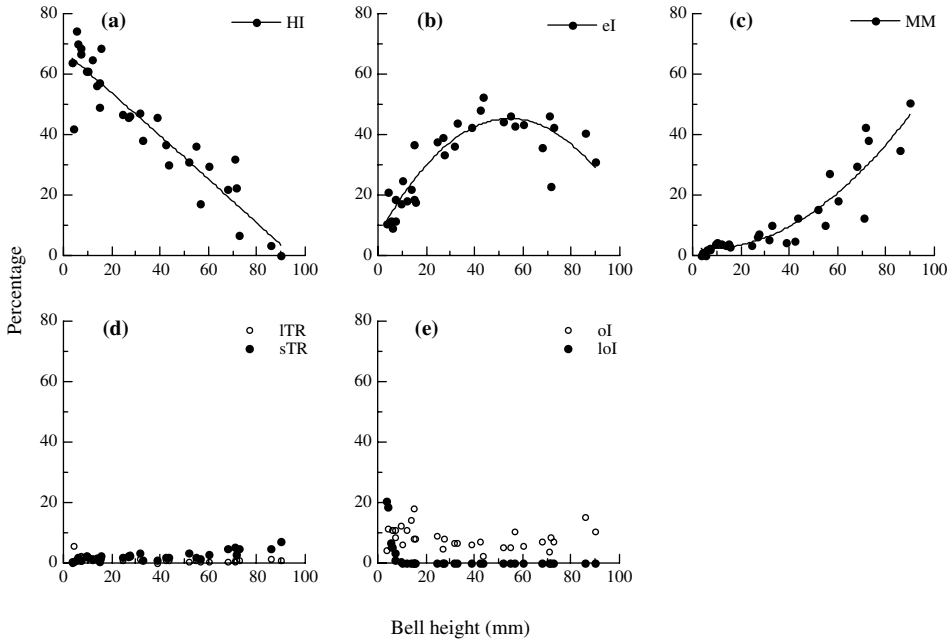

Figure 3. Relative abundance of each type of tentacular nematocyst in *Chiropsalmus quadrigatus* medusae of various sizes. The *X* axis represents bell height (mm) of the medusae and the Y axis represents relative abundance of each type of nematocyst. (a). Holotrichous isorhiza (HI). (b). Ellipsoidal isorhiza (eI). (c). Microbasic mastigophore (MM). (d). Large trirhopaloid (open circles: lTR) and small trirhopaloid (solid circles: sTR). (e). Ovoid isorhiza (open circles: oI) and large ovoid isorhiza (solid circles: loI). The lines represent curves obtained by least-square fit to linear (a) or polynomial (b and c) functions.

Carrette et al., 2002). For example, lTR, sTR, HI, and eI (probably the same type as those classified as atrichous isorhiza in *C. fleckeri*) were present in both *C. fleckeri* and *C. quadrigatus*. However, *C. quadrigatus* was different from *C. fleckeri* in that it possessed ME, oI, and loI, and that MM showed no apparent size classes. Although the nematocyst complements of chirodropid species are similar to each other as suggested by Williamson et al. (1996), they also show some species-specific differences, as shown by Carrette et al. (2002).

This study also showed that nematocyst composition of *Chiropsalmus quadrigatus* changes with body size. Tentacles are used for prey capture, so the changes in the dominant types of nematocysts in tentacles may reflect changes in prey type. HI, the dominant type in tentacles of small individuals, may be used to capture zooplankton, as we observed crustacean plankton entangled by discharged HI in the gut of a small specimen (data not shown). So we guess that small individuals of *C. quadrigatus* prey on crustacean plankton and other small organisms with projections or bristles. MM, one of the predominant nematocyst types in tentacles of large individuals, is thought to have penetrating and injecting functions (Williamson et al., 1996), so we infer it may penetrate and inject venom into relatively large prey such as small fish. eI, which is similar to atrichous isorhizas of *C. fleckeri* at the light microscopic level, may have an adhesive function like atrichous isorhizas of *C. fleckeri* and be used for holding prey that are immobilized by the venom of MM.

Life cycles have been described in four cubozoan species: *Tripedalia cystophore* (see Werner et al., 1971), *Carybdea alata* (see Arneson, 1976; Arneson & Cutress, 1976), *Carybdea marsupialis* (see Studebaker, 1972), and *Chironex fleckeri* (see Yamaguchi & Hartwick, 1980). These studies showed that tentacles of polyps were absorbed and tentacles of medusae were newly formed. At least in *C. alata* and *C. marsupialis*, tissues of medusae such as bell and tentacles are newly formed during metamorphosis and their nematocyst complements differ from those of the polyp stage. If metamorphosis in *Chiropsalmus quadrigatus* occurs in a similar manner as in these species, loI in tentacles, nematocyst clusters on pedalium bases, and sTR on the bells of small medusae may not be remnants of the polyp stage. These nematocysts, which were characteristic of small medusae, might have special functions such as defense during the early medusa phase.

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A review of zoanthid nematocyst types and their population structure

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Key words: Cnidaria, Hexacorallia, *Palythoa, Protopalythoa*, cnidae, capsules, discriminant analysis

Abstract

Seven different types of nematocyst are detailed and illustrated from the Zoanthidea. We studied the size population structure of nematocyst capsules: how they are affected by preservation, and how and what should be measured. Populations of two types, large holotrichs and *p*-mastigophores, from the mesenterial filaments of *Protopalythoa heliodiscus* and *Pr. mutuki* have been compared using length, width, and (for *p*-mastigophores) shaft length. The analysis employed bivariate scattergrams and discriminant analysis. The two species are strongly differentiated by their holotrichs, less well by their *p*-mastigophores. The problem of bimodal size distributions is considered and resolved. It is recommended that nematocyst capsule population descriptors should always be included in species' descriptions, in addition to or instead of the customary minimum–maximum range.

Introduction

The presence of cnidae is one of the defining characteristics of the phylum Cnidaria. Within the Anthozoa, the types, characteristics, and capsule dimensions of one category of cnidae, the nematocysts, have been used in systematics for a century (Fautin, 1988), but the most appropriate data for species discrimination and higher level taxonomy remain open to debate. In this paper we first review the types of nematocyst recorded in Zoanthidea, updating the surveys of Schmidt (1972, 1974), and then explore the use of quantitative data, following the procedural recommendations recently formulated (Ryland et al., 2003). For recognition of nematocyst types, of which at least seven are known for Zoanthidea (Fig. 1), we follow Schmidt (1972, 1974), because of his clear diagrams (Fig. 1), but prefer a terminology based on that of Weill (1934). The first group of types comprises: large holotrichs (holotrichs I of Schmidt) or holotrichous isorhizas (i.e. with parallel sided tubule; Fig. 1D); small holotrichs (holotrichs II) which, from Schmidt's depiction of a tapering tubule, appear to be holotrichous anisorhizas (Fig. 1B and C); and large atrichs – not included by Schmidt in the zoanthid assemblage although they had been recorded from the order by Cutress (1955) (Fig. 1A) and are present in the Actiniaria, from one division of which (Endomyaria) Schmidt (1974) derived the Zoanthidea. The second group comprises the various mastigophores: nematocysts with slender elongate capsules which, following England (1991) – who broadened Weill's (1934) original description to include mastigophores in which the shaft is wider, but only slightly wider, than the rest of the tubule – we identify as basitrichs (Fig. 1E and F; microbasic *b*-rhabdoids of Schmidt); *p*-mastigophores (*p*-rhabdoids of Schmidt), with a wide, sharply defined shaft (Fig. 1G); and microbasic *b*-mastigophores *sensu stricto* (special *b*-rhabdoids of Schmidt; Fig. 1H) which, in zoanthids, are more ovoid and less fusiform than basitrichs, and which – according to Schmidt (1974) – are restricted to the 'higher' Zoanthidea (a term of his own which

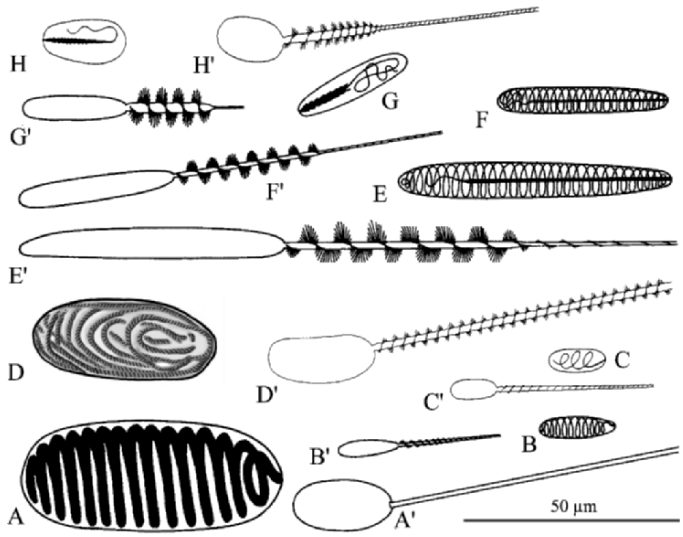

Figure 1. The types of nematocyst found in zoanthids. (A, A′) Atrich, *Palythoa caesia*. (B, B′) Small holotrich (holotrich II), *Palythoa tuberculosa*. (C, C′) Small holotrich, *Parazoanthus axinellae*. (D, D′) Large holotrich (holotrich I), *Palythoa caesia*. (E, E′) Large basitrich, *Palythoa tuberculosa*. (F, F′) Medium basitrich, *Palythoa tuberculosa* (a form with a short shaft occurs in *Parazoanthus axinellae*). (G, G′) Microbasic *p*-mastigophore, *Palythoa tuberculosa*. (H, H′) Microbasic *b*-mastigophores, *Parazoanthus axinellae* (H), *Zoanthus coppingeri* = *pacificus* (H′). Except A′ and D, from Schmidt (1974).

we equate with all the Macrocnemina plus part (family Zoanthidae *sensu stricto*) of the Brachycnemina (see Ryland et al., 2003, for discussion). Schmidt evidently perceived these 'special' *b*-mastigophores as different from those of anemones.

To a small extent, higher anthozoan taxa may be characterized by their types of cnidae, as Hexacorallia are by spirocysts and Ceriantharia by ptychocysts. Carlgren (1940) believed that Ceriantharia were also characterized by lacking *p*-mastigophores, but they are present in arachnactis larvae (Schmidt, 1974). Schmidt considered that zoanthid holotrichs differed from those of all other orders in having 'spoon-shaped' spines, and that 'special' *b*-mastigophores were unique to 'higher' Zoanthidea. Certainly, we have not seen these *b*-mastigophores in the Sphenopidae (equivalent to Schmidt's 'lower' Zoanthidea) which are, however, the only zoanthids to have large basitrichs – of the type prevalent in Endomyaria – in the filaments.

In a comprehensive study of the cnidom of one zoanthid, *Acrozoanthus australiae* (Brachycnemina Zoanthidae), Ryland et al. (2003) demonstrated the scope for error if species' nematocyst capsule sizes are expressed solely as a range between observed upper and lower limits based on a sample of, say, $n = 20$. We investigated the practicalities of using standard statistical procedures to describe nematocyst samples representing populations (as by Hidaka, 1992; Pires & Pitombo, 1992; Chintiroglou & Simsiridou, 1997), rather as done for anemones by Williams (1996, 1998, 2000) but not always reaching the same conclusions. However, there are obvious limitations to the utility of conclusions based on a single species, and we here consider some data from species of Brachycnemina Sphenopidae (Schmidt's, 1974 'lower' Zoanthidea).

Material and methods

For a few specimens, at the Australian Institute for Marine Science (AIMS), fresh nematocysts were obtained as aqueous squashes of fragments of tentacles and filaments. Discharge was encouraged with a drop of acetic acid and pressure on a cover slip, and the preparation viewed under Nomarski illumination. Photomicrographs were taken on colour film, using a ×40 objective and ×3.3 or ×5 photo-ocular; the camera automatically imprinted a scale. Measurements were made from the film, as below.

Later, preserved zoanthids fixed in seawater formalin (4% HCHO), Bouin's fluid, or 70% ethanol were used. After dab drying, since polyp size may be an important variable, volume was determined by displacement (all methods being described in more detail by Ryland et al. (2003)). Using a dissecting microscope, minute amounts of tissue were removed from filaments using extra-fine jewellers' forceps. The tissue was digested for 2–3 min in a few drops of 2% aqueous $KMnO_4$ on a microscope slide, excess fluid absorbed with a strip of filter paper, a drop of glycerol added, and the preparation macerated with the forceps (the method modified from England (1987)). The fragmented cells were spread by light, even pressure on the cover slip, 22×22 mm, which was then ringed with clear nail varnish to prevent evaporation.

Slides were systematically scanned for nematocyst capsules from the top left to the bottom right of the coverslip. All capsules lying wholly in the focal plane were measured; capsules oblique to the focal plane, being foreshortened, were ignored. When available, 100 capsule measurements were made of length, width and, for *p*-mastigophores, shaft length using bright-field or phase contrast illumination and ×40 objectives. Except in the case of the freshly obtained holotrich/atrich sample, we have not used results when $n < 20$. A video camera attached via a ×6.7 photo-ocular fed images to a dedicated image-analysis computer. Following calibration, measurements in μm were made on the screen image. The excellent but now obsolete DOS software MeasurementTV (Updegraff, 1990) was used at the time; we currently use Image-Pro Plus. Original basic statistics were performed in Quattro-Pro, later transferred to MS Excel® 2000 with XLstat add-in (Fahmy & Aubry, 2002) or BIOMstat 3.3 (Rohlf & Slice, 2002) for ANOVA.

Results

When freshly discharged nematocysts from the filaments of *Palythoa caesia* Dana were examined, it was observed that the large, oblong-oval (in plan view) capsules had discharged two different types of tubule. In one, the tubule was wide, ~4.5 μm, with large spines arranged in a triple helix. Undischarged, the tubule was loosely and obliquely coiled, in the manner of a vertical coil of rope that has been pulled sideways from the top, with the spines producing a banded appearance under Nomarski illumination (Fig. 1D). This type corresponded to Schmidt's (1974) holotrich I, described from *Pa. tuberculosa*, although he illustrates the spines in a single helix (Fig. 1D′). In the second type (despite the larger capsule, see below) the tubule was thinner, ~3.7 μm, and appeared totally devoid of spines, even when using a ×100 oil immersion objective; prior to discharge the tubule was coiled in the capsule like a spring (Fig. 1A and A′) – very different from the holotrichs. It corresponded with the atrich described by Schmidt (1974) in actinians but not recorded by him in *Pa. tuberculosa* or any other zoanthid, although he had evidently overlooked that Cutress (1955) had mentioned them from unspecified zoanthids as well as actinians. Our specimens matched Cutress' description perfectly. The holotrich and atrich capsules were also readily separated by size and shape: holotrichs ($n = 4$) length 61.18 (SD ± 2.59) μm, width 26.85 (±1.66) μm, ratio 2.28 (±0.05); atrichs ($n = 10$) length 82.80 (±2.93) μm, width 34.02 (±4.55) μm, ratio 2.47 (±0.29). In a much larger, preserved sample ($n = 100$) of holotrichs the dimensions were 62.29 (±2.78) × 28.60 (±2.08) μm; no atrichs were present. We retain the term atrich, since no spines are visible under the light microscope, though aware that Westfall (1965) – using transmission electron microscopy – could identify small spines around the tubule in the atrichs of *Metridium*.

Photographs were also taken of discharged and undischarged basitrichs and *p*-mastigophores of *Protopalythoa mutuki* Haddon & Shackleton from the rocky shore at Kissing Point, Townsville, for later comparison with preserved material. Using measurements from the film we compared discharged and undischarged basitrichs by ANOVA, the mean lengths being not significantly different: means (±SD) undischarged 57.69 (±3.68), discharged 56.70 (±3.68), $p \approx 0.2$, but width had slightly increased: undischarged 6.69 (±1.00), discharged 7.12 (±0.89), $p = 0.045$. Three methods of fixation were compared: sea water Bouin's fluid for 48 h or more followed by washing and storage in 70% ethanol, direct immersion in 70% ethanol, and 4% formaldehyde in sea water

(though later samples were washed and transferred to 70% ethanol). The dimensions for basitrichs, holotrichs, and *p*-mastigophores are in Table 1. While the long, slender basitrichs (Fig. 1E and F) may perhaps be adequately characterized by length, *p*-mastigophores are quite oval in plan view and holotrichs are oblong-oval (Fig. 1G and D respectively). We have, therefore, measured both length and width. ANOVA of preserved basitrich lengths showed significant differences ($p = 2.67 \times 10^{-9}$), with a similar (though less pronounced, $p = 0.003$–0.0001) result for length and width together using XLstat for discriminant analysis. Moreover, when measurements of fresh undischarged and exploded basitrichs were included, the differences were even more marked ($p < 0.0001$ in all tests). For clarity in the diagram, all the preserved samples have been combined (Fig. 2). On holotrich lengths the corresponding ANOVA was significant ($p = 6.54 \times 10^{-6}$) but discriminant analysis of lengths and widths gave mixed results ($p < 0.0001$–0.12 according to test). There was a different result for *p*-mastigophores with ANOVA indicating no differences in length ($p = 0.168$) but discriminant analysis gave values of $p = 0.002$–0.095. Since we used separate whole polyps, the results do not necessarily indicate that method of fixation produces different degrees of shrinkage, and the three methods have not produced a consistent pattern for the three nematocyst types (Table 1); whether or not fixation method is important requires verification using a partitioned polyp fixed in three ways. The shrinkage caused by any method of fixation is, however, very marked in contrast to the fresh material, exploded or not (Table 1).

The remaining results, all based on preserved material, investigate differences between two sympatric species of *Protopalythoa* from the Great Barrier Reef and Fiji archipelago: *Pr. mutuki* and *Pr. heliodiscus* Ryland & Lancaster (2003). Full details of the types of nematocyst occurring in these two species have been tabulated elsewhere (Ryland & Lancaster, 2003: Tables 2 and 3). Since basitrichs are absent from *Pr. heliodiscus*, our present comparisons are restricted to holotrichs and *p*-mastigophores, which are abundant in the filaments of both species. Holotrich samples of *Pr. mutuki* came from 12 polyps from five sites and of *Pr. heliodiscus* from 11 polyps from seven sites; *p*-mastigophores of *Pr. mutuki* came from seven polyps from five sites and of *Pr. heliodiscus* from 11 polyps from seven sites (Table 1). Additionally, the clearly visible, sharply demarcated shaft in the capsule of *p*-mastigophores (Fig. 1G) can readily be measured, providing a third variable (Table 1).

Bivariate data are conveniently displayed in scattergrams (Figs 3 and 4) but not readily analysed by ANOVA or other univariate methods. Moreover, because two related variables are involved, regressions conform to Model II (Sokal & Rohlf, 1995) and cannot be compared by ANCOVA, although comparisons between correlation coefficients are possible (Sokal & Rohlf, 1995). This situation is discussed in detail, with respect to *Acrozoanthus australiae*, elsewhere (Ryland et al., 2003). Confidence ellipses (normally the 95%) for each bivariate mean (see Sokal & Rohlf, 1995) were calculated in BIOMstat and added to the Excel graph (Fig. 3); these ellipses are analogous to the confidence limits of a single mean (Fig. 4). The separation between the species based on filament holotrichs is particularly clear (Fig. 3), that for *p*-mastigophores less so, largely on account of an anomalous *Pr. mutuki* site (7 in Fig. 4). With two or three variables, discriminant analysis was used. As would be predicted from the plot, a comparison of sites shows significant differences between them. Although the pooled data show a strong difference between the two species ($p < 0.0001$, reinforcing the validity of their separation based on other characters), the amount of overlap would make it difficult to identify any particular specimen on this criterion alone. Of 1606 individual capsule measurement sets, ~16% of *Pr. heliodiscus* capsules would be confounded with *Pr. mutuki*, and ~26% of *Pr. mutuki* would be confounded with *Pr. heliodiscus*. While shaft length (as percentage of capsule length – arcsine transformed in the data analysis but not in the graph) – rather than capsule width is used for the *y*-variate in Fig. 4, because it appeared to be proportionally longer in *Pr. heliodiscus*, the scattergram shows that this is true only for two sites (3 and 8 in Fig. 4).

The statistical analysis of the capsule measurements showed further complications. Just as we had found in *Acrozoanthus australiae* (Ryland et al., 2003), occasional populations of *Pr. mutuki*

Table 1. Measurement data for nematocyst capsules from filaments of two species of *Protopalythoa*, arranged by type

Nem. type	Sp #	Polyp vol. (ml)	*n*	Treatment	Mean length ± SD (μm)	Mean width ± SD (μm)	Shaft L ± SD (μm)	Locality and identification	Loc #
Bas	1	NR	100	Form	48.09 ± 2.49	5.77 ± 0.45	–	Kissing Pt 101/529	5
Bas	1	NR	100	Fresh, expl	56.70 ± 3.23	7.12 ± 0.89	–	Kissing Pt, Townsville	5
Bas	1	NR	23	Fresh, unex	57.69 ± 3.68	6.69 ± 1.00	–	Kissing Pt, Townsville	5
Bas	1	NR	100	Ethanol	49.29 ± 2.49	6.19 ± 0.45	–	Kissing Pt 101/529	5
Bas	1	NR	100	Bouins	46.51 ± 3.83	5.37 ± 0.49	–	Kissing Pt 101/529	5
Hol	1	0.20	100	Form	50.01 ± 2.05	27.11 ± 1.71	–	Caloundra, Qld 106/9613	7
Hol	1	0.10	100	Ethanol	54.39 ± 4.96	22.27 ± 1.71	–	Heron I., GBR 140/9669	6
Hol	1	NR	100	Bouin	50.35 ± 3.68	22.20 ± 2.48	–	Kissing Pt 101/529	5
Hol	1	0.09	100	Bouin	55.98 ± 4.15	28.90 ± 2.63	–	Kissing Pt 101/9642	5
Hol	1	NR	100	Ethanol	51.72 ± 2.63	22.42 ± 2.04	–	Kissing Pt 101/529	5
Hol	1	NR	100	Form	49.51 ± 3.09	22.37 ± 2.12	–	Kissing Pt 101/529	5
Hol	1	0.06	25	Form	47.54 ± 4.70	22.01 ± 1.90	–	Korotogo, Fiji 4/965	11
Hol	1	0.75	100	Form	49.08 ± 2.68	19.16 ± 1.33	–	Korotogo, Fiji 4/961	11
Hol	1	0.11	100	Bouin	55.89 ± 5.65	23.41 ± 3.15	–	Torres Strait 527/9630	1
Hol	1	NR	100	Bouin	53.42 ± 3.89	24.36 ± 2.32	–	Torres Strait 527/202	1
Hol	1	0.10	100	Bouin	49.61 ± 2.78	19.88 ± 1.40	–	Torres Strait 528/9638	1
Hol	1	NR	100	Bouin	47.37 ± 3.57	20.24 ± 1.88	–	Torres Strait 528/104	1
Hol	2	0.11	100	Form	42.63 ± 5.04	16.66 ± 2.13	–	Fitzroy I., GBR 111/9653	3
Hol	2	0.08	26	Form	40.01 ± 5.80	15.45 ± 1.99	–	Fitzroy I., GBR 111/9657	3
Hol	2	0.12	30	Form	41.42 ± 4.62	16.44 ± 2.34	–	Low Is, GBR 210/9651	2
Hol	2	0.25	100	Ethanol	42.58 ± 4.71	20.28 ± 2.69	–	Makuluva Pass 66/9619	9
Hol	2	0.32	100	Ethanol	40.31 ± 3.84	19.35 ± 1.91	–	Makuluva Pass, Fiji 66/9616	9
Hol	2	0.11	90	Ethanol	41.55 ± 4.08	19.67 ± 1.84	–	Makuluva Pass 66/9617	9
Hol	2	0.13	78	Ethanol	40.34 ± 4.13	18.71 ± 2.63	–	Orpheus I., GBR 125/9633	4
Hol	2	0.25	100	Form	40.91 ± 4.92	19.91 ± 2.39	–	Toberua Pass, Fiji 3/966	10
Hol	2	0.15	62	Form	39.25 ± 4.43	19.34 ± 2.39	–	Toberua Pass, Fiji 3/9610	10
Hol	2	0.10	50	Ethanol	34.90 ± 5.98	16.65 ± 2.31	–	Torres Strait BB 1824	1
Hol	2	0.07	100	Form	37.89 ± 4.04	16.26 ± 1.63	–	Yarawa reef, Fiji 43/96101	8
Hol	2	0.08	90	Ethanol	34.65 ± 5.01	15.63 ± 2.29	–	Yarawa reef 44/9701	8
p-Mas	1	0.10	100	Ethanol	22.54 ± 1.95	5.09 ± 0.54	10.28 ± 1.04	Heron I., GBR 140/9669	6
p-Mas	1	NR	100	Ethanol	21.56 ± 1.54	4.91 ± 0.42	NR	Kissing Pt 101/529	5
p-Mas	1	0.09	52	Bouin	22.92 ± 1.56	5.49 ± 0.47	11.34 ± 0.93	Kissing Pt 101/ 9642	5
p-Mas	1	NR	21	Fresh, unex	26.17 ± 1.80	6.46 ± 0.74	NR	Kissing Pt, Townsville	5
p-Mas	1	NR	100	Form	21.89 ± 1.39	4.78 ± 0.29	10.72 ± 0.97	Kissing Pt 101/529	5

Continued on p. 184

Table 1. (Continued)

Nem. type	Sp #	Polyp vol. (ml)	*n*	Treatment	Mean length ± SD (μm)	Mean width ± SD (μm)	Shaft L ± SD (μm)	Locality and identification	Loc #
p-Mas	1	NR	100	Bouin	21.52 ± 1.60	4.71 ± 0.44	NR	Kissing Pt 101/529	5
p-Mas	1	0.10	49	Form	19.66 ± 1.45	4.80 ± 0.49	9.51 ± 1.01	Torres Strait 528/9638	1
p-Mas	1	0.11	52	Bouin	22.40 ± 1.58	4.73 ± 0.34	9.85 ± 0.97	Torres Strait 527/9630	1
p-Mas	1	NR	100	Bouin	18.67 ± 1.51	5.01 ± 0.46	NR	Torres Strait 528/104	1
p-Mas	1	NR	100	Bouin	21.73 ± 2.01	4.81 ± 0.46	NR	Torres Strait 527/202	1
p-Mas	2	0.11	53	Form	27.97 ± 2.45	5.24 ± 0.69	14.81 ± 1.45	Fitzroy I., GBR 111/9653	3
p-Mas	2	0.08	51	Form	27.81 ± 2.12	5.17 ± 0.60	15.60 ± 1.40	Fitzroy I., GBR 111/9657	3
p-Mas	2	0.11	100	Ethanol	27.81 ± 1.85	5.10 ± 0.45	12.47 ± 1.12	Makuluva Pass 66/9617	9
p-Mas	2	0.32	100	Ethanol	27.07 ± 1.54	4.76 ± 0.44	12.56 ± 1.05	Makuluva Pass 66/9616	9
p-Mas	2	0.25	100	Ethanol	26.81 ± 1.98	4.91 ± 0.48	12.06 ± 1.67	Makuluva Pass 66/9619	9
p-Mas	2	0.13	100	Ethanol	25.01 ± 2.36	4.74 ± 0.47	11.94 ± 1.41	Orpheus I., GBR 125/9633	4
p-Mas	2	0.15	100	Form	23.90 ± 1.67	5.15 ± 0.64	11.83 ± 1.29	Toberua Pass, Fiji 3/9610	10
p-Mas	2	0.25	100	Form	23.65 ± 2.10	4.83 ± 0.68	10.44 ± 1.45	Toberua Pass, Fiji 3/966	10
p-Mas	2	0.10	50	Ethanol	25.00 ± 2.10	5.17 ± 0.49	11.30 ± 1.25	Torres Strait BB1824	1
p-Mas	2	0.07	100	Form	22.51 ± 1.10	4.70 ± 0.47	13.02 ± 1.06	Yarawa reef 43/96101	8
p-Mas	2	0.08	100	Ethanol	27.41 ± 1.65	6.21 ± 0.71	12.96 ± 0.83	Yarawa reef 44/9701	8
p-Mas1	1	0.75	100	Form	20.68 ± 1.45	3.83 ± 0.45	8.91 ± 0.72	Korotogo, Fiji 4/961-1	11
p-Mas2	1	0.75	100	Form	42.29 ± 4.42	6.49 ± 1.16	17.68 ± 1.97	Korotogo, Fiji 4/961-2	11
p-Mas2	1	0.26	100	Form	40.56 ± 3.69	6.84 ± 0.69	17.22 ± 1.98	Korotogo, Fiji 4/963	11
p-Mas[1]	1	0.20	100	Form	28.22 ± 3.63	6.24 ± 1.05	13.95 ± 1.57	Caloundra, Qld 106/9613	11

Two *p*-mastigophore samples, one for each sub-population, were taken from one of the Korotogo (4/961). Note 1: the Caloundra sample is the full sample, before separation into components (Fig. 5). Nem. type (nematocyst types): Bas, basitrich; Hol, holotrich; *p*-Mas, *p*-mastigophore. Species are 1, *Pr. mutuki*, 2, *Pr. heliodiscus*; NR, not recorded; *n* = sample size; form, seawater formalin, often later stored in ethanol; expl, exploded; unex, unexploded. Locality numbers (loc #) refer to Figures 3 and 4. In the Korotogo sample there are two populations of *p*-mastigophores: *p*-Mas1, *p*-Mas2.

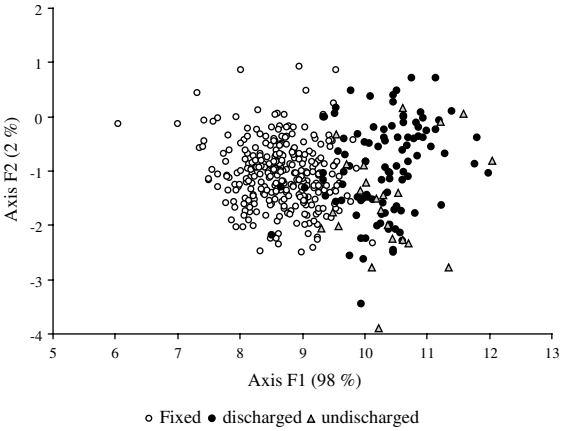

Figure 2. Discriminant analysis of capsule measurements (length, width) of filament basitrichs from *Protopalythoa mutuki* from the rocky intertidal of Kissing Point, Townsville. The comparison is between fixed material (Bouins fluid, 70% ethanol, and 4% sea water formaldehyde, $n = 100$ each, with the samples merged), and un-fixed discharged ($n = 100$) and undischarged ($n = 23$) nematocysts (see text).

(but not of *Pr. heliodiscus*) contained two separate populations of *p*-mastigophores in their filaments. In one sample (Korotogo reef, Fiji; 11 in Fig. 4) the frequency distributions of capsule length were totally separated: p_1 ($n = 96$) mean 20.68 (SD $\pm$ 1.45), range 16.58–24.02 μm; p_2 ($n = 100$) mean 42.95($\pm$3.07), range 35.96–50.61 μm. In a second population (Caloundra, coast of southern Queensland; 7 in Fig. 4), the length-frequency ranges overlap resulting in a bimodal distribution

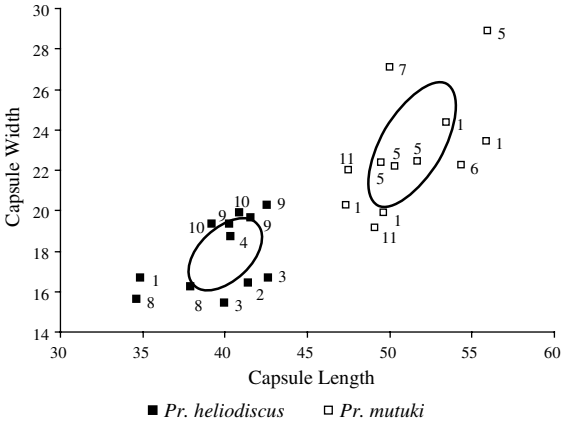

Figure 3. Scattergram, filament holotrich capsule sample means of width versus length in two *Protopalythoa* species, with ellipses indicating 95% confidence limits of the mean of means. Labels are localities (see Table 1 for key).

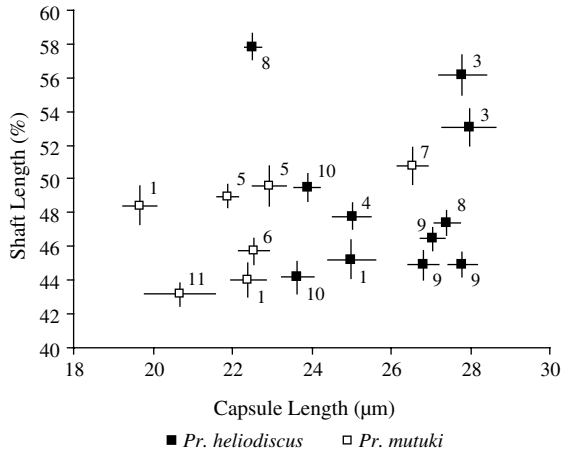

Figure 4. Scattergram, filament *p*-mastigophore capsule shaft length (as percentage of capsule length) versus capsule length sample means in two *Protopalythoa* species. Labels are localities (see Table 1 for key). Error bars are 95% confidence limits.

(Fig. 5). In the diagram we have separated the two components by the standard graphical method (Harding, 1949; Cassie, 1954), reconstituting two separate population distributions (on the assumption of normality) using the proportions of the normal distribution curve found in standard statistical tables (e.g. Zar, 1996). We describe the

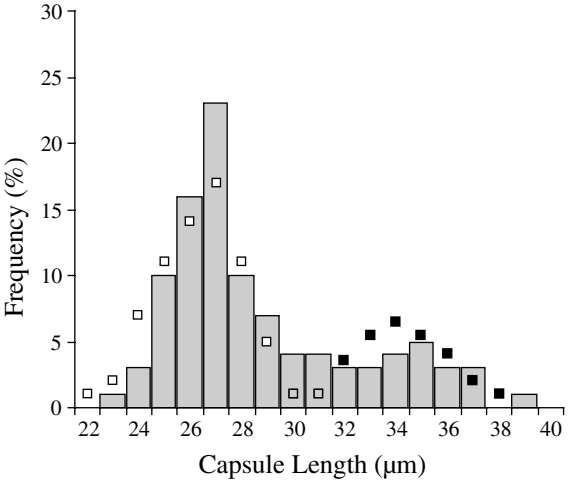

Figure 5. Analysis of bimodal distribution (filament *p*-mastigophores) of *Protopalythoa mutuki* from Caloundra, southern Queensland (grey bars), with the calculated distribution plots of the two sub-populations, p_1 □ and p_2 ■, assuming normal distributions (see text). The 30–31 and 31–32 μm bins contain not only the same number of original measurements but equal numbers of each sub-population. Arbitrary division has therefore been made at 31 μm (see Fig. 6).

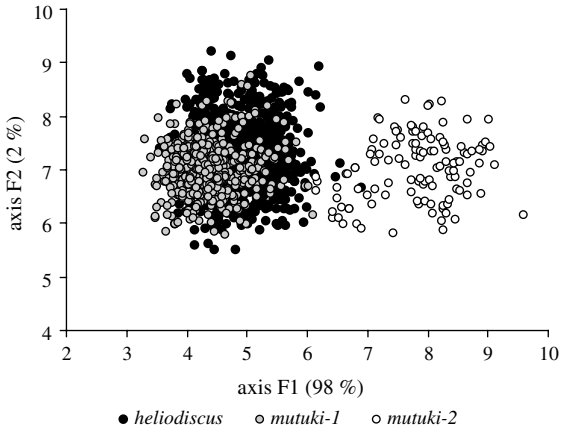

Figure 6. Discriminant analysis of filament *p*-mastigophore capsule measurements (length, width) from two species of *Protopalythoa*, using both p_1 and p_2 sub-populations found in *Pr. mutuki* (see Fig. 5 and text).

method fully elsewhere (Ryland et al., 2003). The method makes no subjective assumptions and shows, in this case, that there is a slight overlap at the tails of the distribution between 30 and 32 μm. Since the two relevant 1 μm bins happened to contain the same number of measurements, and the predicted occurrence of the two types was approximately the same, an arbitrary separation was made at 31 μm. As seen in Figure 4, the point for sample 7 (based solely on the smaller, p_1, population) is still anomalously high; but the analysis establishes that this is *not* the result of sample contamination. The overall result of discriminant analysis, including all *p*-mastigophores (Fig. 6), shows a very strong separation of the p_2 size-group and would almost certainly have indicated if there had been contamination of any other p_1 samples.

Our analyses showed that several of the capsule length–frequency distributions departed from normality. One possible cause of this, as just indicated, would be the inclusion of a few measurements from a distinct, but smaller or larger, population. In other cases the cause of the departure remains unexplained. The overall problem of non-normality is not further considered here, having been thoroughly discussed elsewhere (Ryland et al., 2003).

Discussion

The greatest problem affecting our knowledge of nematocyst types within zoanthid higher taxa is the lack of ultra-structural studies, and hence ability to be able to differentiate types precisely; also, so few species have yet been studied in detail that generalization is unwise. Apart from the identity of 'atrichs', all zoanthid *b*-mastigophores and holotrichs II require SEM investigation. Treating nematocysts as populations depends on the availability of an image-analyser, since sample sizes are ideally at least 50 (Ryland et al., 2003). Digital images are more readily obtainable now but not all imaging software (as the University of Texas' shareware ImageTool) easily permits more than one measurement per object: we consider it desirable to measure as many capsule variables as practicable. Much information can be conveyed in bivariate scattergrams (Figs 3 and 4). The samples can be analysed by multivariate methods, such as discriminant analysis (Figs 2 and 6). The examples given in this paper demonstrate the utility of this approach in revealing differences between species, although inter-colony variation must also be properly evaluated. Discriminant analysis can help to resolve the identity of any particular collection with reference to known species and to decide whether two morphotypes constitute separate species even when capsule size ranges overlap (Fig. 6). The measurements from more than one nematocyst type can be combined in any analysis, increasing the resolving power. The erratic occurrence of differently sized sub-populations, particularly of *p*-mastigophores, is curious, observed in such unrelated species as *Acrozoanthus australiae* (Ryland et al., 2003) and *Protopalythoa mutuki*. Normality must be checked, and bimodal distributions separated into component parts.

Substantial shrinkage of capsules during fixation, such as we have demonstrated, would be expected but the effects of different fixation and preservation protocols – which we have been unable to resolve here – require further investigation. We have not considered in this paper any correlation of nematocyst size with polyp size, a very real consideration in zoanthids, but draw attention to the inconsistent patterns found in *Acrozoanthus australiae* (Ryland et al., 2003): polyp size must always be recorded. So far as species descriptions are concerned, size ranges (i.e. minimum to maximum) should be replaced, or at least supplemented, by calculated statistical parameters.

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Variability in nematocysts from a single individual of the sea anemone *Actinodendron arboreum* (Cnidaria: Anthozoa: Actiniaria)

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Abstract

The taxonomic value of nematocyst size in sea anemones is still being assessed. We evaluate size distribution of nematocysts of one type in a single individual anemone. Length of unfired nematocysts was measured along the column, tentacles, and actinopharynx of a preserved specimen of *Actinodendron arboreum* (Quoy & Gaimard, 1833). Mean, range, minimum, and maximum length of nematocysts vary along the column, those in the middle region being least variable. The length of nematocysts in mature (split) acrospheres is less variable than in immature (unsplit) acrospheres. There is significant variability between nematocysts in tentacles of the primary and quaternary cycles, and along a tentacle, the middle being least variable. Size distribution of actinopharynx nematocysts is complex. The results of this study suggest that assembling data on nematocysts from multiple individuals for taxonomic purposes should be used with an awareness that sampling site can be an important variable. Ideally, the position of tissue sampled should be documented, an attempt should be made to be consistent in sampling from the same position in individuals being compared, and the variability of nematocyst length at each sampled site should be assessed. Inferences can also be made on ontogeny from these data; we conclude that an actinodendrid tentacle grows from the base and at the tips of its branches.

Introduction

The taxonomic value of statistical analyses of cnidae measurements in sea anemones has not been fully evaluated (Fautin, 1988; Williams, 1996, 1998, 2000; Acuña et al., 2003). Carlgren (1900) was the first to assert that no description of a sea anemone species is complete without information on its cnidae (Cutress, 1955; Fautin, 1988). It is now an unstated rule that authors of new species of sea anemones provide information about type, number, and minimum and maximum length of cnidae, and number of specimens sampled. However, no species of anemone can be recognized based on cnidae data alone (Fautin, 1988; Williams, 1996).

In an effort to enhance the value of cnidae in distinguishing species, statistical analyses on nematocyst measurements have been used to study the taxonomy of some sea anemones (e.g. Chintiroglou, 1996; Chintiroglou & Simsiridou, 1997; Allcock et al., 1998; Chintiroglou & Karalis, 2000; Watts et al., 2000; Acuña et al., 2003), with assessments at tissue, intra-individual, populational, and specific levels. Williams (1996, 1998, 2000) analyzed measurements of nematocysts from sea anemones of five families in an effort to determine the number of measurements and the statistical methods that would be of greatest taxonomic use. He found differences between measurements of nematocysts from samples of the same tissue and recommended that each

type of tissue should be clearly identified (Williams, 1996).

We extend the sort of analysis Williams did to the family Actinodendridae, a group of tropical Indo-Pacific, shallow-water animals termed the 'Hell's Fire Anemones' (e.g. Hansen & Halstead, 1971) because of their potent sting. The fact that actinodendrids contain only one category of nematocyst, identified by Carlgren (1940, 1949) as basitrichs, helps in the sampling process; no errors can occur in their identification. Because an anemone can grow to be relatively large (as much as 400 mm in length and crown diameter), numerous repetitive tissue samples can be collected with accuracy from multiple sites.

The 48 tentacles of a member of *Actinodendron* – one of the three genera of the family – are ramified in branches that make it resemble a head of broccoli (Fig. 1). As is true for all sea anemones, the tentacles develop in cycles, the six constituting the first cycle forming first, nearest the mouth; they are endocoelic (i.e. they communicate with the spaces between the members of a mesentery pair). The six secondary tentacles, set more marginally, and the 12 tertiary ones, set even more marginally, are also endocoelic. The 24 marginal tentacles (referred to here as constituting the fourth cycle) are exocoelic (i.e. they communicate with the space between members of adjacent pairs of mesenteries). Distinctively, the tentacles ramify fractally, a first-order branch stemming from a

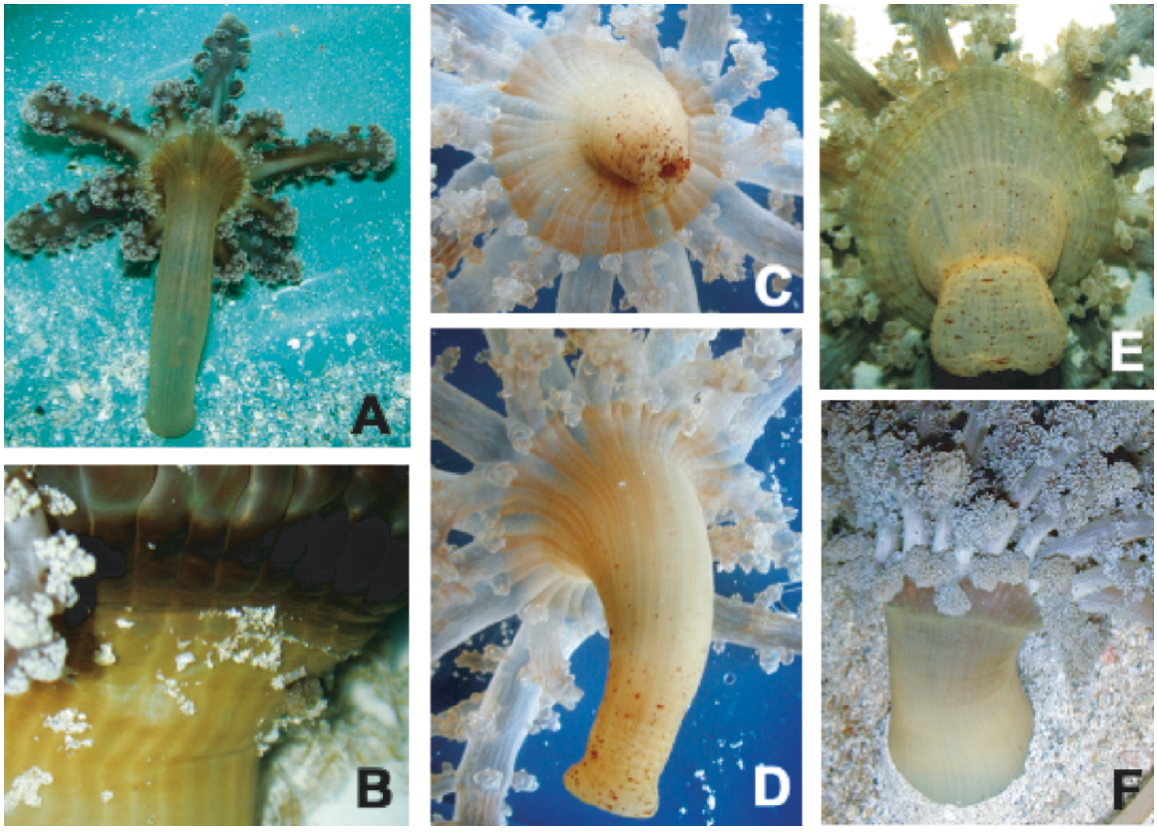

Figure 1. Morphology and coloration of live specimens of *Actinodendron*. The column is widest at the distal end of well-expanded individuals, and commonly it is darker as well. (A) Juvenile of *A. alcyonoideum* (Quoy & Gaimard, 1833) from Fiji (USP 4890). (B) Detail of column of an adult of *A. alcyonoideum* from Fiji (USP 4888). (C and D) Column of a juvenile of *A. arboreum* (Quoy & Gaimard, 1833) from Madang, Papua New Guinea (KUNHM 1671); in C the proximal and middle column are contracted, in D the column is elongated. (E) An individual of *A. arboreum* from Alotau, Milne Bay, Papua New Guinea (KUNHM 1673). (F) Middle and distal column in an individual of *A. arboreum* from Motupore Island, Papua New Guinea (KUNHM 1826). A typical animal is 100 mm long. Photographs by AA.

tentacle, with a second-order branch arising subsequently from a first-order branch, etc. A tentacle of *A. arboreum* can have as many as five series of branches. Each branch ends in a bilobed acrosphere (thickened tissue containing dense nematocysts). We infer the branching is an adaptation for intercepting light, actinodendrids being zooxanthellate.

One of us (AA) studied replicate tissue samples from a single specimen of *Actinodendron arboreum* (Quoy & Gaimard, 1833) from along column, tentacles, and actinopharynx. The objective was to identify the least variable sampling sites within an individual, a desideratum if nematocyst measurements among specimens are to be compared for taxonomic purposes.

Because the regions of high variability in nematocyst lengths in *A. arboreum* coincide with regions (such as the base of tentacles) where Robson (1988) reported cnidogenesis to be active in other anthozoans, we infer that regions in which size of nematocysts is highly variable may be where they are formed. Campbell (1988, p. 117) envisioned that variability of nematocysts of a single type within one species and between species 'will doubtless be important in unraveling developmental mechanisms' of nematocysts. Robson (1988, p. 199) considered that 'differential distribution of the cnidom, often a taxonomic characteristic, reflects on the aspect of cnidogenesis about which almost nothing is known.' Using this criterion, we infer that the tips and base of a tentacle and the distal and proximal regions of the column are possible cnidogenic regions in *Actinodendron*.

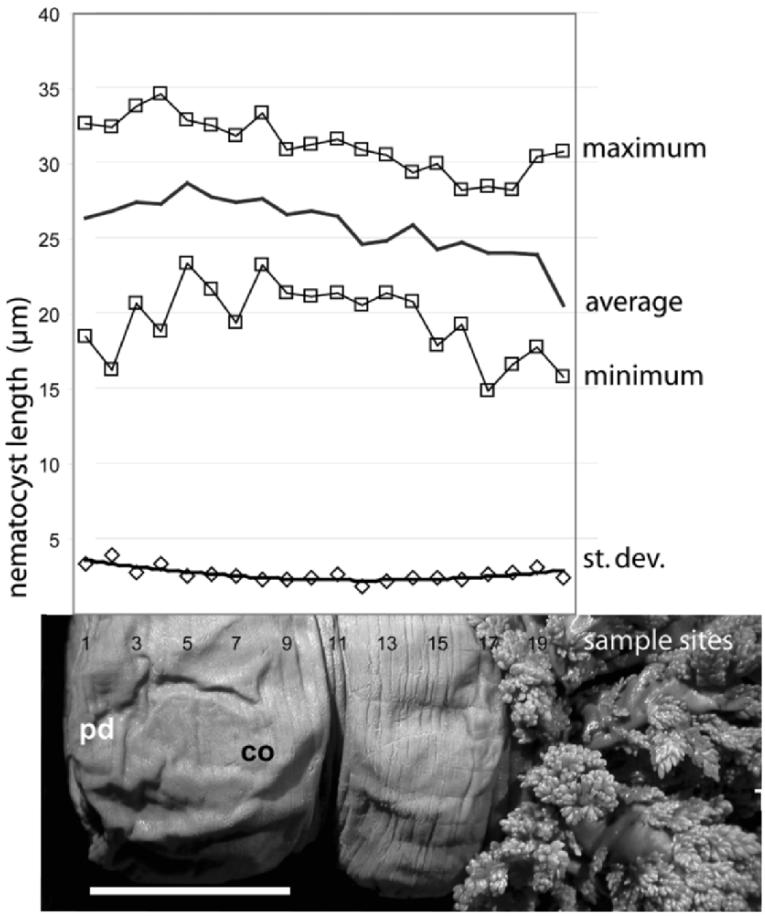

Figure 2. Length of nematocysts in 19 samples along the column of the studied specimen (MNHN 1562) of *Actinodendron arboreum* (Quoy & Gaimard, 1833). pd, pedal disc; co, column; scale bar is 50 mm.

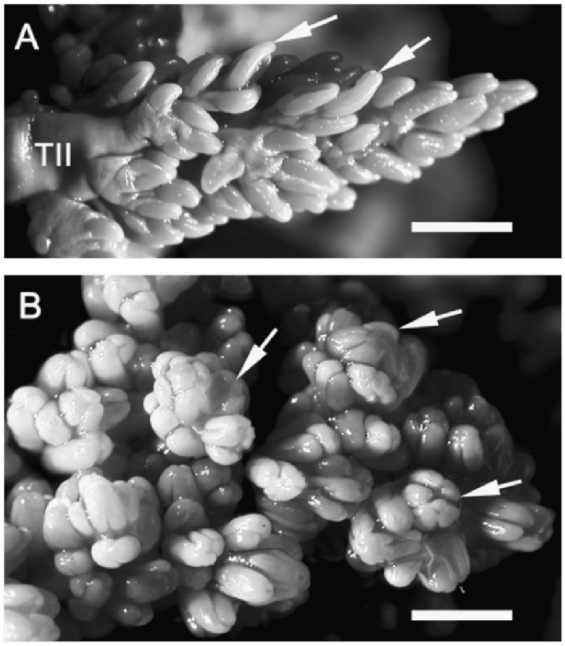

Figure 3. Acrospheres in the studied specimen (MNHN 1562) of *Actinodendron arboreum* (Quoy & Gaimard, 1833). (A) Mature (split) acrospheres; (B) Immature (unsplit) acrospheres at the tip of tentacles. TII, branch of the second order in a first cycle tentacle; arrows indicate the acrospheres. Scale bars are ~2 mm.

Material and methods

Abbreviations: KUNHM – Kansas University Natural History Museum, Lawrence, Kansas (USA); MNHN – Museum National d'Histoire Naturelle, Paris (France); USP – University of the South Pacific, Suva (Fiji)

One whole specimen of *Actinodendron arboreum* (Quoy & Gaimard, 1833) was examined (Fig. 2). It was collected at Baies des Citrones, New Caledonia (Station 109, HZ19) by P. Laboute on 29 July 1980. It is cataloged as MNHN 1562.

AA examined undischarged cnidae at 1000× in squash preparations using a light microscope equipped with differential interference optics. Sigma Scan Pro version 4.01.003 measurement software was used to measure the length of undischarged capsules projected onto a Summa Sketch digitizing tablet (Summagraphics).

Sampling was done following the recommendations of Williams (1996). At least 40 nematocysts were measured from each site studied. Nineteen points were sampled along the column (Fig. 2); 881 capsules were measured. Nematocysts were also measured from: the tip, middle, and base of the aboral side of two tentacles of the primary and two tentacles of the quaternary cycle; four mature (split) acrospheres (Fig. 3A) and four immature (unsplit) acrospheres (Fig. 3B); and seven sites in the actinopharynx, which is long with two large siphonoglyphs that extend into the coelenteron more deeply than the wall of the rest of the actinopharynx (Fig. 4).

Basic statistics were calculated using the software Microsoft Excel, Release 97. General Linear Model, multivariate discriminant analyses, and Andreson-Darling normality tests were calculated using the software Minitab Release 13 (http://www.minitab.com).

Results

Based on fired capsules in another individual of *Actinodendron arboreum*, one of us (AA) determined that the actinodendrid nematocysts referred to by Carlgren (1940, 1949) as basitrichs are

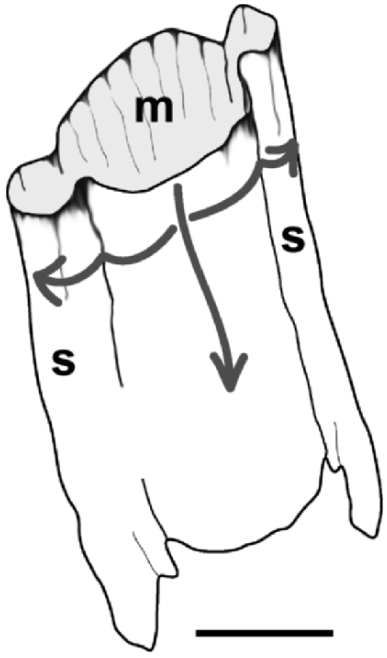

Figure 4. Schematic diagram of an actinopharynx in *Actinodendron arboreum* (Quoy & Gaimard, 1833). Arrows indicates the direction in which the size of nematocysts increases. m, mouth; s, siphonoglyph. Scale bar is ~10 mm.

Table 1. Lengths (in μm) of column nematocysts

Site	*n*	Min	Max	Mean	Median	SD	Range	*p*
1	43	18.5	32.6	26.6	25.9	11.8	14.1	0.470
2	46	16.2	32.4	26.9	27.7	15.3	16.1	0.000
3	46	20.7	33.9	27.5	27.5	7.8	13.1	0.885
4	52	23.4	32.9	28.7	28.7	6.5	9.5	0.057
5	45	21.6	32.6	27.8	28.2	6.9	11.0	0.248
6	46	19.5	31.8	27.5	28.1	6.6	12.4	0.001
7	46	23.3	33.4	27.6	27.6	5.4	10.1	0.387
8	41	21.4	30.9	26.7	27.1	5.4	9.5	0.468
9	48	21.2	35.5	27.0	27.2	7.1	14.2	0.778
10	51	21.4	32.5	26.6	26.6	7.5	11.1	0.618
11	49	20.6	28.4	24.5	24.5	2.5	7.8	0.325
12	57	21.3	30.5	24.9	24.5	4.7	9.1	0.029
13	40	20.8	29.4	26.0	26.4	5.7	8.6	0.045
14	43	17.9	30.0	24.2	24.0	5.9	12.1	0.549
15	42	19.3	28.3	24.7	25.0	5.3	9.0	0.235
16	50	14.8	28.5	24.1	24.2	6.9	13.7	0.468
17	44	16.6	28.2	24.1	24.9	7.7	11.6	0.009
18	46	17.8	30.4	24.0	23.8	9.7	12.7	0.412
19	46	15.8	30.8	20.6	20.5	6.0	15.0	0.050

Sites are those depicted in Figure 3; *n*, number of samples; min, minimum length; max, maximum length; *p*, value of Andreson–Darling normality test.

actually microbasic *b*-mastigophores. There were two categories of them in the specimen studied, narrow thick-walled and wide thin-walled types. The measurements and statistics given here are of the former, which was present in all tissues and in an abundance that made the study practical. With the exception of the oral end of the actinopharynx, all nematocysts of the narrow thick-walled type spanned a single size range in each tissue.

Column

Minimum and maximum length, range, standard deviation, and, with some exceptions, mean and median (Table 1) delimited three regions in the column: proximal (sites 1–8 in Fig. 2), middle (sites 9–14 in Fig. 2), and distal (sites 15–19 in Fig. 2). Using discriminant analysis (Table 2), 16 of 19 data samples (84%) were correctly classified. The squared distance between the groups of the proximal and middle column was smaller (4.2) than that between the middle and distal column (8.2). Gross morphology corresponds at least somewhat to our findings: the distal column in live specimens is thinner and may be colored more intensely than the rest of the column (Fig. 1). The range in length of nematocysts and their standard deviation was larger in the proximal and distal column; the shortest nematocyst was in the distal column, the longest in the proximal column.

Table 2. Summary of classification of basic statistics of length of nematocysts in three regions of the column of a specimen of *Actinodendron arboreum* (Quoy & Gaimard, 1833) as generated by multivariate discriminant analysis

	Proximal-actual	Middle-actual	Distal-actual
Proximal-assigned	7	2	0
Middle-assigned	0	4	0
Distal-assigned	0	1	5
Total	7	7	5
Correctly assigned	7	4	5
Proportion correct	1.00	0.57	1.00

Tentacles

The General Linear Model test with a nested unbalanced design indicated significant effects for type of tentacle (primary cycle and quaternary cycle) ($F = 24.72$, $p \sim 0.000$), for position of tissue sample along a tentacle (tip, middle, and base) ($F = 141.27$, $p \sim 0.000$), and interaction between these two categories ($F = 34.77$, $p \sim 0.000$). Two-sample *t*-tests on samples of the same type of tentacle and same position along the tentacle indicated differences in the tip and base of tentacles. There was no evidence for significant differences among samples from the middle of the same type of tentacle (Table 3).

Acrospheres

Descriptive statistics are presented in Table 4. Discriminant analysis classified the measurements of nematocysts from split and unsplit acrospheres into separate groups ($n = 8$; proportion correct 1.000).

Actinopharynx

Descriptive statistics are presented in Table 5.

Discussion

Because only the repetitive samples from the middle column were invariant in range and minimum and maximum length of nematocysts, we conclude that this region is the most appropriate for sampling nematocysts in comparing measurements among individuals for taxonomic purposes. The proximal, middle, and distal regions we characterize by basic nematocyst statistics may have functional significance. The proximal column is actively used in burrowing and locomotion; the distal column is morphologically distinct, commonly appearing more inflated than the rest of the column and more darkly pigmented (Fig. 1). However, these attributes cannot be perceived in preserved specimens.

There are significant differences among nematocysts sampled from tentacles of primary and quaternary cycles. By ignoring the cycle of a tentacle, an investigator might compare nematocysts from tissues that differ in both topology and age. In actinodendrids as in other sea anemones, it may be difficult to distinguish a tentacle of the primary cycle from one of the secondary or even tertiary cycle. Therefore, in actinodendrids only tissue sampled from the tentacles of a quaternary

Table 3. Lengths (in μm) of nematocysts from tip, middle, and base of the aboral side of two tentacles of the primary and two tentacles of the quaternary cycle; abbreviations as in Table 1

Tentacle cycle	Position	*n*	Min	Max	Mean	Median	SD	Range	*p*	*p* value of *t*-test
First	Base	41	16.1	22.2	18.5	19.7	1.7	6.1	0.198	0.001
		50	16.6	26.7	20.1	20.5	2.1	10.0	0.198	
	Middle	46	15.1	21.8	17.2	17.4	1.8	6.7	0.020	0.432
		43	15.2	21.4	17.6	18.0	1.3	6.1	0.558	
	Tip	41	15.5	18.5	16.6	17.4	0.7	4.9	0.648	0.000
		48	13.6	18.5	15.0	15.6	1.0	10.0	0.198	
Fourth	Base	50	16.2	24.3	18.7	19.2	1.7	8.1	0.815	0.000
		47	17.3	23.8	19.8	20.8	1.7	6.5	0.122	
	Middle	42	15.5	20.9	17.5	18.3	1.3	5.4	0.542	0.497
		50	15.4	20.8	17.4	18.0	1.5	5.3	0.389	
	Tip	45	17.3	21.0	18.6	19.5	0.8	3.7	0.277	0.000
		52	13.8	20.1	16.8	17.6	1.4	6.3	0.653	

Table 4. Lengths (in μm) of nematocysts from four mature (split) acrospheres (Fig. 3A) and four immature (unsplit) acrospheres (Fig. 3B); abbreviations as in Table 1

Maturity	*n*	Min	Max	Mean	Median	SD	Range
Split	43	73.2	102.6	87.9	87.87	7.4	29.5
	47	69.0	102.3	87.0	86.70	7.3	33.3
	52	72.4	107.4	87.4	86.98	7.4	35.0
	44	73.8	107.2	91.6	89.67	7.3	33.4
Unsplit	53	53.7	97.7	80.7	79.90	9.6	44.0
	41	61.5	99.2	82.2	84.44	8.7	37.7
	57	64.4	99.7	87.4	88.95	7.9	35.2
	58	62.7	99.8	81.7	81.65	7.6	37.1

Table 5. Lengths (in μm) of nematocysts from seven sites in the actinopharynx: oral end (two samples), middle, lower middle, and aboral end of a siphonoglyph, and oral, middle, and aboral regions of wall outside the siphonoglyphs (Fig. 4); abbreviations as in Table 1

Site	*n*	Min	Max	Mean	Median	SD	Range
Siphonoglyph oral	45	17.5	34.45	24.0	22.9	4.2	16.9
	40	34.6	49.9	39.8	39.0	3.7	15.2
Siphonoglyph middle	44	36.6	54.3	47.3	47.7	4.3	17.9
Siphonoglyph middle aboral	40	40.1	54.4	47.6	48.4	4.2	14.3
Siphonoglyph aboral	42	31.1	42.8	36.1	36.1	2.4	11.7
Wall oral	45	17.9	33.2	29.0	29.3	2.6	15.3
Wall middle	44	28.6	38.8	33.4	33.3	2.3	10.2
Wall aboral	43	29.6	44.5	37.1	37.1	3.1	14.9

cycle might be suitable for comparisons for taxonomic purposes.

Significant differences can be found in length of nematocysts from various sites along a single tentacle. Nematocysts from the middle of a tentacle of the same cycle varied least among samples. This may be related to function, to ontogeny, or to both.

Robson (1988) reported that a sea anemone tentacle grows from its base and that distally, typically in the region around an apical pore, tentacle tissue disintegrates. Thus, a tentacle cell constantly moves distally during its lifetime. An actinodendrid tentacle cannot grow this way because it is not a simple tapering cylinder like that of a typical anemone. In at least the actinodendrid genera *Actinodendron* and *Megalactis*, branching occurs continually along the length of each tentacle, and the tip of a tentacle branch lacks an apical pore, terminating instead in an acrosphere. Finding that in *Corynactis* sp., a corallimorpharian anthozoan with acrospheres similar in structure to those in actinodendrids (although each tentacle has only one, at its apex), acrosphere tissue contains irregular inclusions and phagocytosed nematocysts, Robson (1988) hypothesized that fired nematocysts may be replaced by nematoblasts that differentiate at the site. Based on differences in the length of nematocysts in mature and immature acrospheres of *A. arboreum*, we infer that acrospheres of actinodendrids also contain cnidogenic tissue. This conclusion is supported by comparative morphology (AA, unpublished). The morphological complexity of an actinodendrid tentacle means that growth of the tentacle is complex, occurring at the tip of each branch as well as its base.

The actinopharynx is a tissue with a complex pattern of nematocyst size distribution: size increases from the oral end toward the coelenteron

and from the middle region toward the siphonoglyphs (Fig. 4). Therefore, the actinopharynx is less suitable than the other structures studied for comparing nematocysts among individuals for taxonomic purposes.

Our results generally agree with those of Williams (1996, 1998, 2000), who found that nematocyst size within a tissue is normally distributed. However, some of our samples had a very low p value with the Andreson-Darling normality test (Tables 1 and 3). Absence of normality in size distribution of some nematocysts has also been found in several acontiarian species by Acuña et al. (2003) and by Ryland (personal communication) in zoanthids.

For taxonomic purposes, sampling sites for nematocysts should be carefully selected and documented. Ideally, it is important not only to be consistent in how sampling is done but to know the variability of nematocyst size within each region. Regions that are more variable may be less suitable for taxonomic or other comparisons among individuals, but they may carry information about development or other ontogenetic processes. The results of this study reiterate what has been shown previously, that quantitative data on nematocysts should be used in sea anemone taxonomy with suitable caveats and in conjunction with other sorts of characters. Perhaps only after the nematocyst size distribution is mapped from at least one individual in each sea anemone family should statistical comparisons among individuals in those families be done.

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Hydroid nematophores: morphological, structural, and behavioural variety from old knowledge and new data

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Abstract

There is a rich old literature on nematothecae, which have been described from many species of nine families of thecate hydroids, but detailed knowledge of nematophores is from a very few species. Using both old data and new observations, information was gathered on species belonging to the genera *Hydrodendron*, *Zygophylax*, *Antennella*, *Plumularia*, *Monotheca*, *Macrorhynchia*, *Gymnangium*, and *Thecocarpus*. Specimens belonging to five families collected on the coral reefs of Réunion Island (SW Indian Ocean) were observed alive and videotaped to gather information on nematophores. Nematophores are classified and named here as belonging to three types. An amoeboid nematophore of the four plumularid families Kirchenpaueriidae, Halopteriddidae, Plumulariidae, and Aglaopheniidae usually consists of two parts, which are more separate in aglaophenids than in the others. The cnidostyle (or nematostyle) is fixed, is formed of ectoderm and endoderm, and includes a cluster of large nematocysts at its top. It is inferred to serve in defence because nematocysts inside the nematotheca can be fired when stimulated. The sarcostyle is a mobile amoeboid layer of ectoderm capable of considerable extension; those that are bilobed on top can extend in two directions at a time. The sarcostyle can emit small pseudopods on its edges that phagocytose particles at the surface of the perisarc. Its hypothesized function of cleaning the colony and providing nutrition in the form of small detrital particles and bacteria taken up by phagocytosis remains to be confirmed by experimentation. The cnidostyle-like nematophore of the Campanulinidae, Linolariidae, and Clathrozoidae is a fixed structure formed of a bilayered pedicel ending with a cluster of nematocysts able to fire while still inside the nematotheca, like the cnidostyle part of the amoeboid nematophore. It functions in defence as well. The tentacle-like nematophore of the Haleciidae and Lafoeidae, described here for the first time, is a single very extensible process provided with nematocysts that issue from the nematotheca and formed by a file of endodermal chordal cells surrounded by ectoderm. In halecids, nematocysts are not always gathered on the very top, forming what looks like a strongly capitate tentacle, as was previously thought, but can be more scattered, giving the structure a filiform appearance. This type has active movements, being able to twist and bend in all directions. Its prey capture function has not been demonstrated; a sensory function is more probable. Additional data have to be collected for more species to compare the types with more confidence, but they appear to have different origins for their considerably different morphologies, structures, and behaviour.

Introduction

According to Cornelius (1995a, p. 332 and 1995b, p. 329), a nematophore is a modified polyp on a hydroid colony which typically, perhaps in all species, lacks a mouth and functions in defence. All are richly armed with nematocysts. Known in many thecate families. There are several recogni-

sed types. Some are protected in thecae termed nematothecae … and some are unprotected, or naked. Nematophores of *Aglaophenia* and perhaps of some related genera have two recognised portions, the sarcostyle (the 'body' of the structure) and the cnidostyle (= nematostyle, the end region bearing the nematocysts).

This precise definition took into account old data lost for nearly a hundred years. The coenosarcal appendages on a corbula were discovered, according to Allman (1864), by Huxley and were named nematophores by Busk because they contain clusters of large thread-cells. Allman (1864) described the nematophores of plumularids as containing an amoeboid protoplasm producing pseudopodia. This discovery was so exciting that numerous scientists became interested in studying the histology of plumularid nematothecae to verify Allman's data. Among the publications during the second part of the 19th century are those by Hincks (1868), Merejskovski (1882) and Pausinger (1899). Hincks (1866) discovered in a halecid species a new structure that he considered equivalent to a plumularid nematophore, and Spencer (1891) found a clathrozonid species provided with nematophores as well. Nutting (1900) did a revision of the knowledge of his time, but during the entire 20th century, data were not added as research focused mainly on the skeleton rather than the soft parts. In fact, descriptions and classification of thecates were based almost entirely on skeletal characters, and numerous taxonomic works hardly mentioned nematothecae. In some of the most important works there is no description of nematophores (e.g. Naumov, 1960; Millard, 1975; Bouillon, 1994; Hirohito, 1995) as there is for more specialized studies on plumularids (e.g. Bedot, 1923; Boggle, 1975; Calder, 1991; Schuchert, 1997).

Nematothecae are found in nine thecate families: Campanulinidae, Lineolariidae, Haleciidae, Lafoeidae, Clathrozoidae, Kirchenpaueriidae, Halopteridiidae, Plumulariidae, and Aglaopheniidae (the last four are commonly called plumularids as they were first considered subfamilies of Plumulariidae). They are present in all genera of the plumularid families and of the Clathrozoidae while, on the contrary, they are present in only a few genera of the other families. Although this represents nearly 40 genera, the morphology, structure, and function of the soft parts, the nematophores, are known for only a very few species studied during the 19th century. They are totally unknown for the Lafoeidae and nearly so for the Campanulinidae, Lineolariidae, and Halopteridiidae.

During the Fourth International Workshop of the Hydrozoan Society (Bodega Bay, USA, 1998), E. A. Robson (Reading University) noted that knowledge of hydroid nematophores comes from very old studies and she reviewed some of these observations (Robson, in prep.). She questioned the validity of those observations and hypotheses. As a preliminary revision of the knowledge, at the beginning of the 21st century, the families will be treated here separately and, where possible, new data collected from living specimens will be compared with the literature. Based on the definition by Cornelius (1995a,b), three nematophores types are described. An entire revision will be done later with more complete data from study of other species and families.

Material and methods

Specimens were collected by scuba diving on the west coast of Réunion Island in the southwest Indian Ocean. They or their substrata were carefully detached and put immediately and separately in plastic bags filled with water. The bags are put in insulated jars for transport to the laboratory. Observations on living animals were done by means of a stereomicroscope (Wild M420) and a compound microscope (Wild Diaplan) as soon as possible. Merejkovski (1882) recommended work not only on alive animals but on animals at ease and absolutely at rest. Therefore, Petri or deeper dishes and depression slides were used. Video sequences were taken. Illustrations are from photographs taken with a Nikon Coolpix 4500 or still images taken from video recordings.

Results

New data were collected for five families: the Clathrozoidae, Kirchenpaueriidae, and Lineolariidae are absent on Réunion Island, and species of

Campanulinidae with nematothecae are rare and were not observed alive.

Families Campanulinidae and Lineolariidae

The two families are treated here together because Lineolariidae was previously included in Campanulinidae. According to Bouillon (1985) and Watson (1992), the presence of nematothecae is a generic character for only four genera of Campanulinidae, *Egmundella*, *Lafoeina*, *Lovenella*, and *Oplorhiza*, and for the two genera of Lineolariidae, *Lineolaria* and *Nicoliana*. These nematothecae are scattered and either tubular (short or long) or globular (Cornelius, 1995a; Hirohito, 1995). However, few details are available about the structure and function of the nematophores that are protected inside. Allman (1883, p. 6) wrote of a 'granular fleshy column, which supports a cluster of thread-cells' in an *Oplorhiza* species, and cited appendages of the same kind in a *Lafoeina* described by Sars. Naumov (1960) figured what looks like a very thin pedicel bearing a large bundle of nematocysts at the top for *Oplorhiza diaphragmata* Naumov. Because of the cluster of nematocysts, they are considered to have a defensive function.

Family Clathrozoidae

The three genera (*Clathrozoella, Clathrozoon*, and *Pseudoclathrozoon*) are all provided with nematothecae. In 1891, Spencer published drawings of *Clathrozoon wilsoni*, a species that he discovered, showing long and thin tubular scattered nematothecae. He represented the nematophores as composed of two parts: a pedicel, with endodermal and ectodermal layers, and a top composed of a large bundle of nematocysts. In his review of the family, Hirohito (1967) wrote that the nematophores of the new genus *Pseudoclathrozoon* were similar in structure to those of *Clathrozoon wilsoni* and of *Clathrozoella drygalskii*, as described by Vanhöffen (1910).

Family Haleciidae

The genera *Hydrodendron* and *Scoresbia* are provided with nematothecae. In the original description of *Ophiodes mirabilis* (now *Hydrodendron*), Hincks (1866, p. 421) described and illustrated well a 'remarkable tentacular organ very extensible with enlarged head containing numerous thread-cells.' He said that this structure 'may be considered as the equivalent of the nematophore,' thereby discovering a new type of nematophore.

Three *Hydrodendron* species have been found on Réunion Island, two with nematothecae on the hydranth pedicel and one with them on the hydrorhiza. The nematophore is a tentacle-like extensible process (Fig. 1a and b) provided with an ectoderm that can fold to contract (Fig. 1e and f) and surrounding a file of large chordal endodermal cells (Fig. 1f and g). It is armed with nematocysts that are either gathered in a cluster at the top – which makes the nematophores look like a strongly capitate tentacle (Fig. 1a and e) – or more dispersed, although densely packed, which makes it look like a strongly filiform tentacle (Fig. 1b and g). In her description of *Scoresbia daidala*, Watson (1969) figured such a captitate nematophore.

Family Lafoeidae

According to Bouillon (1985), nematothecae are present in five genera: *Abietinella*, *Bedotella*, *Cryptolaria*, *Eucryptolaria*, and *Zygophylax*, but no data seem to have been collected on nematophores. Only once were they observed in extension and moving on a living colony of *Zygophylax rufa* (Bale) from Réunion, although specimens were collected several times. While the hydranths were retracted inside the hydrothecae, nematophores were very active. They appeared as filaments that looked like very long filiform tentacles, and were capable of reaching a at least five times the length of a hydrotheca and of twisting and bending in all directions (Fig. 1c and d). They have no distal capitation but the tip can sometimes appear swollen, according to the state of contraction. The structure has not been observed with a microscope.

Family Kirchenpaueriidae

Nematothecae are either absent or reduced in this family but nematophores are present in all species. First knowledge of these nematophores came from the work of Merejkovski (1882). He described the

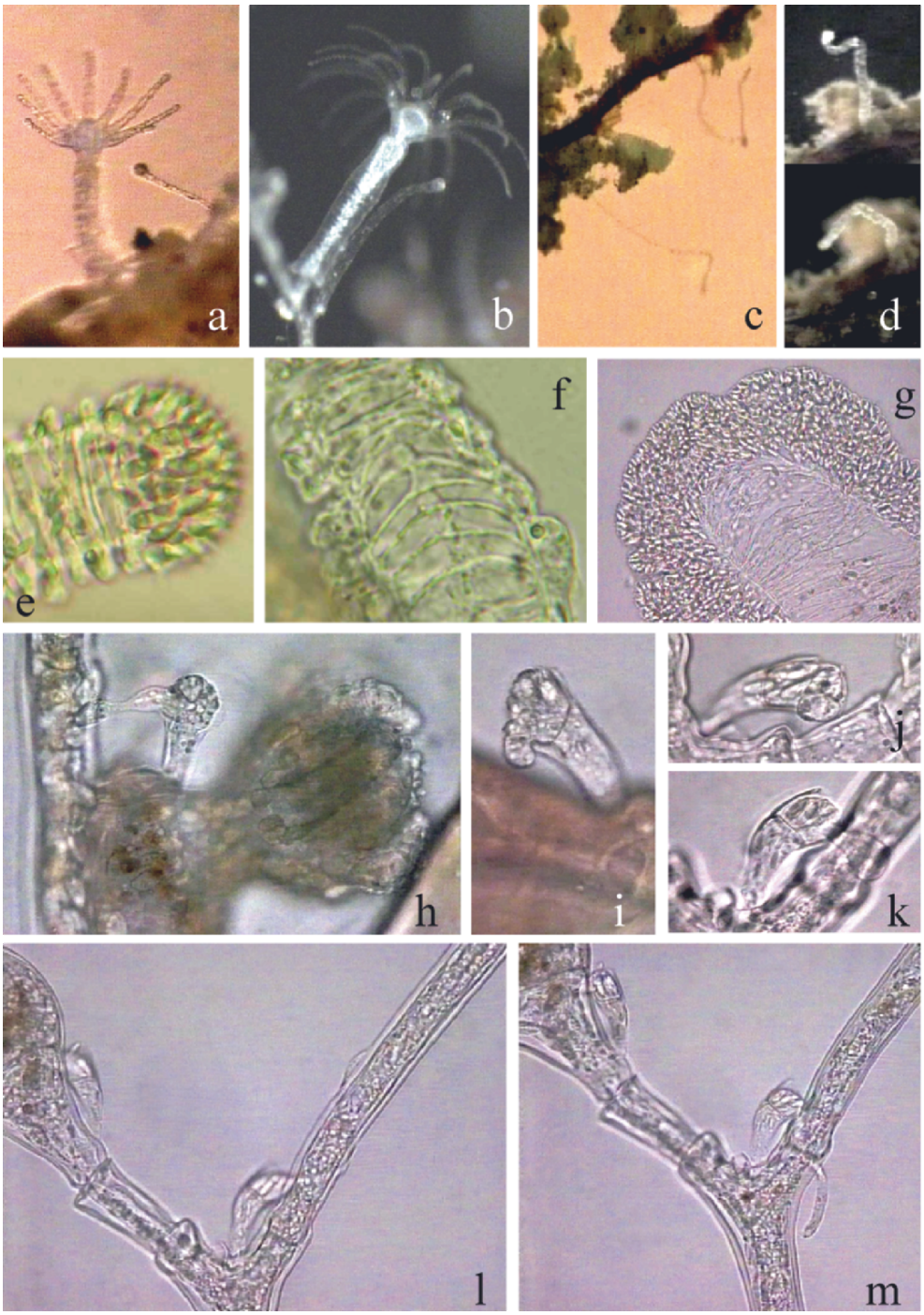

Figure 1. Nematophores, tentacle-like and amoeboid. (a, b, e–g) Haleciidae – two *Hydrodendron* species: hydranths and tentacle-like nematophores (a, b); details: ectoderm folding (e, f), chordal endoderm (f, g), nematocysts in a capitation (e) for (a) and scattered (g) for (b) species. (c, d) Lafoeidae, *Zygophylax rufa*: tentacle-like nematophores on a branch with a hydrotheca (c) and different attitudes (d). (h) Halopteridiidae, *Antennella secundaria*: bithalamic nematotheca with cnidostyle (on right) and expanded sarcostyle stuck to the hydrocladia (on left). (i–m) Plumulariidae, *Plumularia* sp.: mobile bithalamic nematotheca with cnidostyle under and nematostyle above in the distal chamber (i); *Monotheca* sp. nov.: nematophore at rest in a bithalamic nematotheca (j), sarcostyle beginning to expand (k), expanded upwards and twined around the stem (l) and retracting after a downwards expansion (m). (All photographs are from life).

nematophore of *Ventromma halecioides* (Alder) emerging from a reduce nematotheca and formed by an immobile, cylindrical mass composed of ectoderm and endoderm, and of an extremely mobile amoeboid part composed entirely of ectoderm. He demonstrated the cellular contents of this amoeboid process by killing the colony with osmic acid, then staining it with carmin borate, and hypothesised the cells of the amoeboid process were surrounded by intercellular protoplasm. He provided evidence that the nematophores were quite different from those in a plumularid and an aglaophenid species that he studied, although all had an amoeboid part. Nutting (1900, p. 25), studying *Kirchenpaueria pinnata* (Linné), confirmed Merejkovski's observations but conversely said the nematophores were of simpler type, not being divided into nematocyst-bearing and adhesive parts. Nor did he confirm the existence of intercellular protoplasm, saying that 'the ectodermal cells are themselves amoeboid and capable of conjointly exhibiting the wonderful extensibility so often described.'

Family Halopteriididae

Bithalamic nematothecae are present in all genera. Their morphologies were redescribed recently by Schuchert (1997). But nematophores are still unknown. New data are from *Antennella secundaria* (Gmelin) from Réunion Island. There is a common pedicel inside the basal chamber of the lateral nematotheca, but inside the distal chamber the soft content is divided into two parts supported but not protected by the cup-shaped skeleton. The part that is fixed and compact includes small nematocysts; the other is mobile and amoeboid, being able to emerge and to extend long enough to contact with the skeleton of either the hydrocladium or the hydrotheca to which it adheres and onto which it spreads (Fig. 1h). This is also so for the median and cauline nematothecae. These data agree with the figure in Pausinger (1899) for *Halopteris diaphana* (Heller).

Family Plumulariidae

All genera possess bithalamic nematothecae. They have been documented in three *Nemertesia* species. *Nemertesia antennina* (Linné), which was studied by Merejskovski (1882), Allman (1883) and Nutting (1900), has simple nematophores formed of a single extensible part. The two species studied from Réunion Island differ in having nematophores in two parts. Large plumose colonies of *Plumularia* sp. exhibited a clean skeleton. In life, soon after collection, many amoeboid processes were noticed spread on the surface of the perisarc in all parts of the colony. The species had clearly visible mobile nematothecae, each with a mobile sarcostyle and fixed nematostyle (Fig. 1i). Very small colonies of *Monotheca* n. sp. were equally clean. Inside a resting nematophore, the nematostyle containing large oval nematocysts was abcaulinar while the sarcostyle looked rounded and adcaulinar (Fig. 1j). As it began to spread out, it formed a sort of tongue that came into contact with the skeleton of the stem (Fig. 1k), then extended slowly upward, very far from the nematotheca, while remaining in contact with the stem and twining around (Fig. 1l). Extension was also possible downward. Therefore the sarcostyle can sweep over much of the surrounding skeletal stem. To recover to its initial position inside the nematotheca, it contracts while detaching and retracting (Fig. 1m).

Family Aglaopheniidae

All genera possess monothalamic nematothecae. In the first record of a nematophore, Allman (1883) noted that it comprised two parts, one amoeboid and one bearing the thread-cells. Merejkovski (1882) provided very good drawings of *Aglaophenia pluma* (Linné): the fixed nematostyle bears a bundle of nematocysts, while the sarcostyle can expand widely through lateral and basal orifices of the nematotheca. He used acetic acid and carmin borate to demonstrate the cellular structure of the sarcostyle previously supposed to be acellular. Histological observations led him to consider the nematophore solid, without gastrosvascular cavity but with an endodermal axis, the mobile part being exclusively ectodermal. He confirmed the presence of pseudopodia on the edges of the well-expanded sarcostyle, as noted by Allman. He also showed that nematostyle and sarcostyle were more separate in the nematophores of the aglaophenid he studied than in those of the plumularid and kirchenpauerid he studied.

New studies done on Réunion Island on three species of three other genera, *Macrorhynchia philippina* (Kirchenpauer), *Gymnangium gracilicaule* (Jäderholm), and *Thecocarpus brevirostris* (Busk), confirmed old data and found additional ones. Concerning the median nematotheca of *M. philippina* (Fig. 2a–f), separation between the two parts of the nematophore occurs at its very base, the nematostyle being located on the outer face of the nematotheca while the sarcostyle is on the inner, as figured by Merejkovski (1882) for *A. pluma*. The nematostyle is fixed and never emerges from the nematotheca. The nematocysts inside are at the very top, grouped in a bundle, the cnidocils probably reaching the terminal opening and so being able to induce firing (Fig. 2c). The sarcostyle emerges through the basal orifice of the nematotheca and spreads over the outer surface of the hydrotheca (Fig. 2a). This ectodermal amoeboid process is composed of cells, as it sometimes carries away pigmented cells (Fig. 2b). It is obviously bilobed at rest, being able to spread in two directions at a time, a morphology until now unknown (Fig. 2e–f). This is not the case for the sarcostyle of the lateral nematotheca that, however, can spread all over the nematotheca or on the surrounding skeleton (Fig. 2h–j). Both can elongate greatly and they fold as well when they contract to retract inside the nematotheca (Fig. 2f and j). As described for other species, the sarcostyle is able to emit on its edges pseudopodia, visible under great magnification, that may include granules of various sizes (Fig. 2g), and sometimes very small and unidentified nematocysts have been observed too. Nematophores of the phylactocarps are of the same type, the amoeboid sarcostyle expanding towards and over the next nematotheca.

The nematophores of the two other aglaophenid species observed were similar although not identical. The sarcostyle of the median nematotheca of *T. brevirostris* was bilobed and the nematostyle comprised several very obvious glandular cells (Fig. 2k). Such cells were previously described as granular cells in *A. pluma*. They were present also in cauline nematothecae, in which the nematostyle was double in respect to the two superior orifices (Fig. 2l). In *G. gracilicaule*, the sarcostyle of the median nematotheca, which is probably not bilobed, was seen expanding into the hydrotheca through the basal orifice with which it communicates (Fig. 2m). Its structure looks like that figured by Warren (1908) for *G. segmentata* (Warren), and a similar behaviour was noticed in *A. pluma* by Allman (1864) and Pausinger (1899).

Discussion

Morphology, structure, and behaviour

It emerges from this preliminary revision that the term 'nematophore' includes very different structures: single or double, very mobile or partly or totally fixed. Due to their different structures and morphologies, I propose to classify the nematophores into three types: tentacular-like, amoeboid, and cnidostyle-like. All of them, except in a few Kirchenpaueriidae species, are partly or entirely protected by skeletal components called nematothecae. Nematothecae are present in nine thecates families. Their distribution in a colony is most precise in the four plumularid families, less precise in lineolarids, halecids, and lafoeids, and least precise in campanulinids and clathrozonids.

Tentacular-like nematophores occur in families Haleciidae (*Hydrodendron, Scoresbia*) and Lafoeidae (*Zygophylax*). They are simple and, like hydranth tentacles, are composed of an internal file of large chordal endodermal cells surrounded by a thin ectoderm. The nematophore is very extensible, the ectoderm being able to shrink and fold while contracting; it is provided with nematocysts either distributed along the distal part or gathered into a capitation on top; it is very mobile, being able to move in various directions, and to bend and fold down.

Amoeboid nematophores are supposed to be found in all genera of the four plumularid families (Kirchenpaueriidae, Halopteridídae, Plumulariidae, and Aglaopheniidae) because they possess nematothecae similar to those in the few species already studied. A nematophore is usually a double structure, composed of a cnidostyle and a sarcostyle. The cnidostyle (or nematostyle), composed of endoderm and ectoderm, often includes granular cells that seem to be glandular, and ends in a bundle of nematocysts. It is fixed, and the nematocysts on top can discharge, although they are inside the nematotheca. The sarcostyle is an ectodermal amoeboid process. It is mobile,

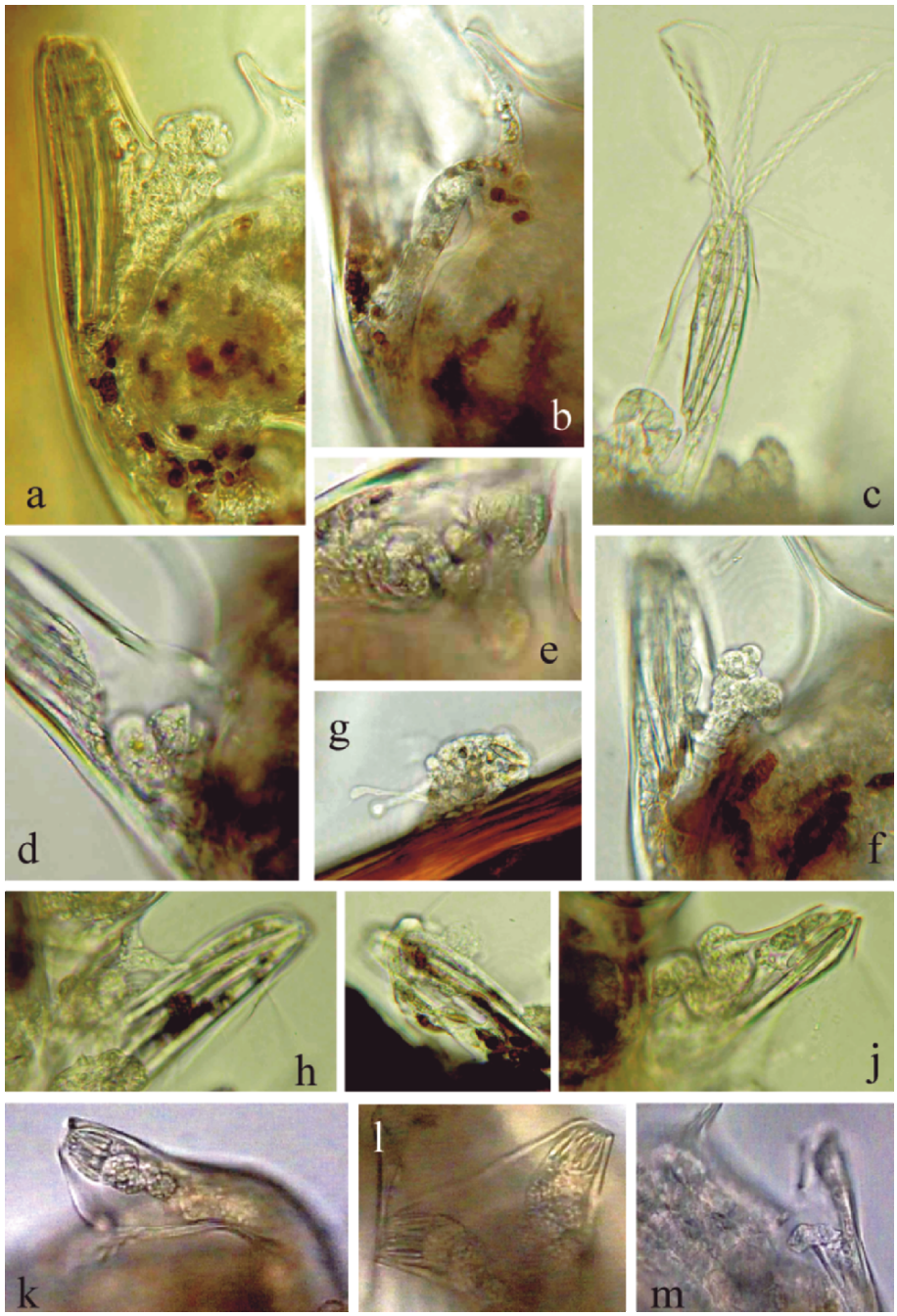

Figure 2. Amoeboid nematophores. (a–m) Aglaopheniidae, *Macrorhynchia philippina* (a–i): median nematotheca (a–f, g): cnidostyle with nematocysts undischarged (a) and discharged (c); sarcostyle entirely retracted (d), expanded and stuck on the hydrotheca (b), engaged inside the basal orifice while expanding (a) or retracting with basal folds in profile (f) and ventral (e) views, bilobed (e, f); lateral nematotheca (h–j): cnidostyle with undischarged nematocysts and sarcostyle expanded towards the hydrocladium (h), all around the nematotheca and bearing coloured cells (i) and retracting (j); pseudopodia on distal part of a sarcostyle where particles and a small nematocyst are included (e). (k, l). *Thecocarpus brevirostris*: median nematotheca: bifid sarcostyle entirely retracted basally and distal nematostyle with gland cells and cluster of nematocysts (k); cauline nematotheca with two nematostyles (sarcostyles below, not visible). (m) *Gymnangium gracilicaule*: median nematotheca with sarcostyle expanding inside the hydrotheca. (All photographs are from life).

although slow-moving, and very extensible; it usually expands while remaining in contact with the skeleton. While extended, it can produce on its edges very thin pseudopodia, and it is able to phagocytose small granules on the surface of the skeleton. The two parts of an amoeboid nematophore are not identical in all the families as they are more or less developed. In the Halopteriddidae and Plumulariidae, for example, the cnidostyle and sarcostyle are supported by the same pedicel and are side by side in the upper chamber of the bithalamic nematotheca, unprotected. (Fig. 1h–j). By contrast, in the Aglaopheniidae they are separated from the base of the nematotheca, and both are protected inside the monothalamic nematotheca (Fig. 2d and f). In this family, the sarcostyle spreads outside through the basal orifices of a nematotheca, and that of a median nematotheca can also expand inside the hydrotheca. It is bilobed in some genera, being able to extend in two directions at a time. Conversely, some species of the Kirchenpaueriidae and Plumulariidae, although having amoeboid behaviour, have been described as lacking a well-separated cnidostyle and sarcostyle. Additional data are needed for a comprehensive survey for these families.

Cnidostyle-like nematophores are found in Clathrozoidae (*Clathrozoon*, *Clathrozoella*, *Pseudoclathrozoon*), Campanulinidae (*Egmundella*, *Lafoeina*, *Lovenella*, *Oplorhiza*), and Lineolariidae (*Lineolaria*, *Nicoliana)*. From the few data available, a nematophore in all the families is thought to be a simple structure resembling the cnidostyle of an amoeboid nematophore in having a pedicel formed of endoderm and ectoderm (this needs to be verified for campanulinids) and in ending with a bundle of nematocysts. It is fixed, the nematocysts being able to discharge while inside the nematotheca.

Function

Although no experiments or careful studies were done during the 19th century to understand nematophore function, several hypotheses were offered. Owing to the presence of nematocysts inside, they were all supposed to be modified defensive polyps. This looks logical for the cnidostyle-like nematophores of campanulinids, lineolarids, and clathrozonids, and for the cnidostyle of the compound nematophores of the four plumularid families. Owing to their capacity to move and twist in all directions, tentacle-like nematophores of halecids were also supposed both to play a role in prey capture ('purveyor' of Hincks, 1866) and to be sensory, 'tasting' the water of the surroundings. It seems logical that those of lafoeids are sensory too. When seen in action, the hydranths were, on the contrary, retracted inside the hydrothecae, so were not in a position to feed, and nothing but the skeleton of the colony was to be defended. Nutting (1900) thought the aglaophenid sarcostyle possibly to function in attaching the leaves of young corbulae to others at the end of this skeletal structure. Moreover, he proposed that sarcostyles scavenge, as he observed them 'particularly active in the vicinity of degenerating hydranths and mutilated gonangia' (Nutting, 1900, p. 25, Fig. 88). Allman (1864) wrote of a cleaning function, an hypothesis supported by the fact that aglaophenid and plumularid colonies are always clean while, conversely, colonies of other families (lafoeids and sertularids, for example) usually bear a lot of epizoans like diatoms, algae, crustaceans, or polychete tubes. New data from Réunion Island confirm that sarcostyles contain particles that probably come from the surface of the skeleton to which they adhere. These particles could be removed by the amoeboid processes as they spread, being engulfed by phagocytosis. They could be used in nutrition, as suggested by Hincks (1868) for *Plumularia setacea* (Linné) and *P. frutescens* Ellis and Solander. Thus the amoeboid nematophores of plumularids could have three different functions: defence by the cnidostyle, and nutrition and cleaning by the sarcostyle. Moreover, a function of regeneration was also suggested, as the sarcostyle retracting inside the hydrotheca and coalescing with the remnant tissues of a degenerated hydranth has been observed (Merejkovski, 1882; Pausinger, 1899).

Conclusion

For the Haleciidae, new data confirm the observations of Hincks (1866) and provide additional information on the presence of non-capitate nematophores in other *Hydrodendron* species. Nematophores of Lafoeidae are briefly described for the first time in the genus *Zygophylax* and are considered similar to those of Haleciidae, although much longer. Further studies are needed to inves-

tigate the supposed sensory function of this tentacle-like type of nematophore. Concerning Halopteriididae, new data on a species of *Antennella* confirm previous observations from a species of *Halopteris* (Pausinger, 1899): there is a fixed nematostyle (cnidostyle) and a mobile sarcostyle. In Plumulariidae, old data were contradictory. New data from *Plumularia* and *Monotheca* show amoeboid nematophores with abcauline cnidostyles and adcauline sarcostyles side by side; previous data on *Antennularia* were of ameboid nematophores not divided into two parts. The sarcostyles of *Monotheca* species are greatly extensible. For Aglaopheniidae, new data confirm those from *Aglaophenia* and *Gymnangium*, and bring in information on *Macrorhynchia* and *Thecocarpus*. Morphological differences among these genera and according to location of the nematothecae (mesial, lateral, or caulinar) in a single species were obtained for the first time.

Additional data are needed for all families, specially campanulinids, lineolarids, and lafoeids. Studies with SEM and TEM are needed to be precise about morphology and structure, and to infer function in order to compare the nematophore types. Then comparison is needed with the dactylozoids of athecates, known to be defensive polyps, but which usually contain a gastrovascular cavity. Many questions about nematophores remain. Are those of Kirchenpaueriidae formed of one (Nutting, 1900) or two (Merejkovski, 1882) parts, or both? How can the sarcostyle of a plumularid extend so widely and pass from such a contracted to such an expanded state, being a single-layered ectodermal process? If it is true that the tentacle-like nematophores of halecids and lafoeids are sensory, under what conditions do they act, and what sort of information do they bring to the colony?

Because their morphologies, structures, and functions differ so much, it seems the three nematophore types could not have the same origin. They are strictly distributed in the colonies of plumularid families, which have amoeboid nematophores, while, on the contrary, they are distributed at random in campanulinids and clathrozonids, which have cnidostyle-like nematophores, and there are some preferential sites for those of halecids and lafoeids, which have tentacle-like nematophores. For the amoeboid type, old studies demonstrated that the development was different between aglaophenids and plumularids (Merejkovski, 1882). It would be interesting to enlarge such studies to other species, being precise about morphological and structural differences among the four families of plumularids *sensu lato*. Halecids have often been treated as near plumularids. The present discovery of tentacle-like nematophores in lafoeids like those in halecids, and the wide difference between such nematophores and those of plumularids is in contradiction with this point of view.

It was previously noted that terminology is confusing (e.g. Nutting, 1900): the term 'nematophore' has been used for single soft bodies (Allman, 1864) or for the skeletal envelope, or for both (Allman, 1883). As knowledge grows, it is important to take care that a term is clearly understandable and also to clarify and keep the history of term usage and synonyms. To build a consensual and illustrated list of terms is the main goal of a recently-begun collaborative thesaurus project in which specialists are involved. They will discuss the validity of terms proposed here for the various nematophores types. To begin, the question will be to decide, with so different structures and functions, if it is right to term them all nematophores only because they include nematocysts. Then they will need to consider Hincks' opinion (1866) that the extensible organ he discovered on halecids (tentacule-like) was equivalent to plumularid (amoeboid) nematophores.

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Part IV
Taxonomy & Systematics

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Use of X-radiographs to distinguish members of the *Montastraea annularis* reef-coral species complex

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Key words: Montastraea, coral reef, Pleistocene, coral banding, species complex

Abstract

Recent work suggests the *Montastraea annularis* species complex consists of at least three species, which can be distinguished qualitatively in the field using features related to colony growth (e.g. overall growth form, bumpiness, growth along the colony edge). However, when whole colonies are not available and surfaces are eroded, identification becomes problematic when relying on such characteristics. Characters based on internal skeletal structures are less prone to loss due to taphonomic processes. Previous work has shown that internal corallite architectural features measured in transverse thin sections can be used to distinguish species. To determine whether internal colony-level features measured on X-radiographs can be used, eight characters related to corallite budding and accretionary growth were measured on specimens representing three modern members of the *M. annularis* species complex (*M. annularis*, *M. faveolata* and *M. franksi*), as well as two fossil forms (columnar and organ-pipe). All eight characters showed significant differences among species. Discriminant function analysis using seven of these characters resulted in distinct species groupings in canonical scores plots and a 100% classification success for specimens from Panamá. These results suggest that measurements made on X-radiographs provide a useful tool for quantitatively distinguishing members of the *M. annularis* complex as well as between other massive reef corals.

Introduction

Montastraea annularis sensu lato is one of the dominant reef-building corals in the Caribbean and is found essentially on all Caribbean reefs over a wide range of depths (Goreau & Wells, 1967; Logan, 1988; Van Veghel & Bak, 1993). For the past several decades, *M. annularis* was considered to be a single species that exhibited considerable morphological variation, with much, though not all, of that variation being associated with differences in light availability (Dustan, 1975; Graus & Macintyre, 1976, 1982). Recently, work by Knowlton et al. (1992) led to the conclusion that *Montastraea annularis* is actually three distinct species and the consequent re-recognition of two previously described species, *M. franksi* and *M. faveolata* (Weil & Knowlton, 1994).

With the breakup of *M. annularis* into at least three species comes the need to distinguish these species in both modern and fossil materials. Means of separating the different species include molecular techniques, growth rates, aggression, ecology, corallite structure, whole colony morphology, transverse thin-sections, and surface-skeletal characteristics (Graus & Macintyre, 1982; Budd, 1993; Budd et al., 1994; Weil & Knowlton, 1994; Van Veghel & Bosscher, 1995; Budd & Johnson,

1996; Van Veghel et al., 1996; Budd & Klaus, 2001; Knowlton & Budd, 2001; Pandolfi et al., 2001, 2002). Unfortunately, many of the conventional techniques used to distinguish members of the *Montastraea annularis* species complex are ineffective in distinguishing fossilized specimens. Species determination in fossilized material is especially difficult when relying on 3-dimensional morphometric characteristics because corallite surfaces are easily eroded, (Budd & Klaus, 2001; Knowlton & Budd, 2001; Pandolfi et al., 2002). Likewise, full, intact colonies are not readily available for many fossil specimens or for core samples, so growth form is not always known. Further complications with fossilized materials arise from the presence of additional species, or morphotypes, during the Pleistocene co-occurring with modern day members of the complex (Budd & Klaus, 2001; Pandolfi et al., 2001, 2002).

As the internal structure of coral colonies is much less prone to weathering than surficial features, identification based on internal-skeletal characteristics may be more diagnostic for fossilized specimens. Internal corallite structures, examined in transverse thin-sections, have been found useful in differentiating the species (Budd & Klaus, 2001); however, internal structures reflecting whole colony morphology have not been examined. X-ray analysis allows for the examination of density differences within skeletal slabs (Macintyre & Smith, 1974). X-rays are commonly used for growth rate measurements by examining annual density bands; however, the use of X-rays to examine other skeletal details related to colony growth has not been investigated extensively (Macintyre & Smith, 1974; Graus & Macintyre, 1976, 1982; Knowlton et al., 1992; Darke & Barnes, 1993). By examining additional skeletal details, measurements made on X-radiographs may be able to serve as proxies for whole colony morphology, and thus serve as useful indicators of species.

Methods

Sampling

Collections of both modern and fossil coral specimens were analyzed. Forty-eight living coral specimens from the San Blas Islands, Panamá, were collected from patch reefs at depths between 3 and 12 m from 1995 to 1997 (Table 1). The specimens were identified in the field as belonging to the three recently described species of the *M. annularis* complex based on the following characteristics: (1) *M. annularis* sensu stricto (Ellis & Solander, 1786), which forms multiple smooth columns with diameters usually 100–350 mm and senescent colony edges, (2) *M. faveolata* (Ellis & Solander, 1786), which forms smooth to keeled mounds (single mound-shaped skeletal mass with a diameter >350 mm) with non-senescent edges, and (3) *M. franksi* (Gregory, 1895), which forms bumpy mounds and plates (single and multiple) with non-senescent edges. The field-based identifications were subsequently confirmed by molecular analyses (Fukami et al., in press), and corroborated by transverse thin section morphometrics (Knowlton & Budd 2001; Pandolfi et al., 2002).

Table 1. Number of each species (living colonies) or growth form (Pleistocene colonies) collected from each location and total numbers

Species or growth form	Panamá	Belize	Barbados	Total
M. annularis	14	15	–	29
M. faveolata	9	5	–	14
M. franksi	25	6	–	31
Columnar	–	–	27	27
Organ pipe	–	–	30	30
Total	48	26	57	131

Only living colonies were collected in Panamá and Belize, and only fossil colonies were collected in Barbados.

Twenty-six living coral specimens from various reef settings at Carrie Bow Cay, Belize, were collected from depths between 0.5 and 30 m from 1970 to 1975 (Table 1); most were previously analyzed by Graus & Macintyre (1982). The specimens were identified as belonging to the three recently described species of the *M. annularis* complex based on the gross appearance (shape) of colonies in X-radiographs.

In Barbados, 57 fossil coral specimens were collected from an 82 000 year old reef terrace (Table 1) (specimens from Pandolfi et al., 2002). In Barbados, only the organ-pipe and columnar growth forms described by Pandolfi et al. (2001, 2002) were analyzed and these were collected from a shallow water (<5 m) back-reef setting. Organ-

pipe colonies were defined qualitatively as composed of numerous elongate skeletal pipes whose diameters were usually less than 100 mm thick; in contrast, columnar colonies are composed of multiple skeletal columns (higher than wide) with diameters usually 100–350 mm thick. Both of these forms differ morphologically from modern *M. annularis*; however, only the organ-pipe growth form has thus far been considered a distinct, extinct species (Pandolfi et al., 2002). Both modern *M. annularis* and fossil-columnar forms have columns lacking living corallites along their sides, while the organ-pipe form had living corallites along the column sides (Pandolfi et al., 2002).

Morphological characters

Specimens were cut through the top of the colony, parallel to the growth axis, into ~5 mm thick slabs. Slabs were X-rayed and the negatives digitized using a Umax Power Look II scanner with transmitted light. Image-Pro Plus v. 3.1 was used to record the morphological measurements. Eight characters were measured from the digitized X-ray images (Figs 1, 2; Table 2).

Wherever possible, measurements were made on five replicate corallites or sections of each colony for all characters, except growth angle and maximum angle for which only one determination was made. For specimens on which five replicate measures could not be made of a given character, as many measurements as possible were made. For some specimens certain characters could not be measured.

Data analysis

For each coral colony, replicate measurements of each characteristic were averaged before being used in statistical analysis. Pearson correlation coefficients were calculated for each pair of characters. Significant differences between species and

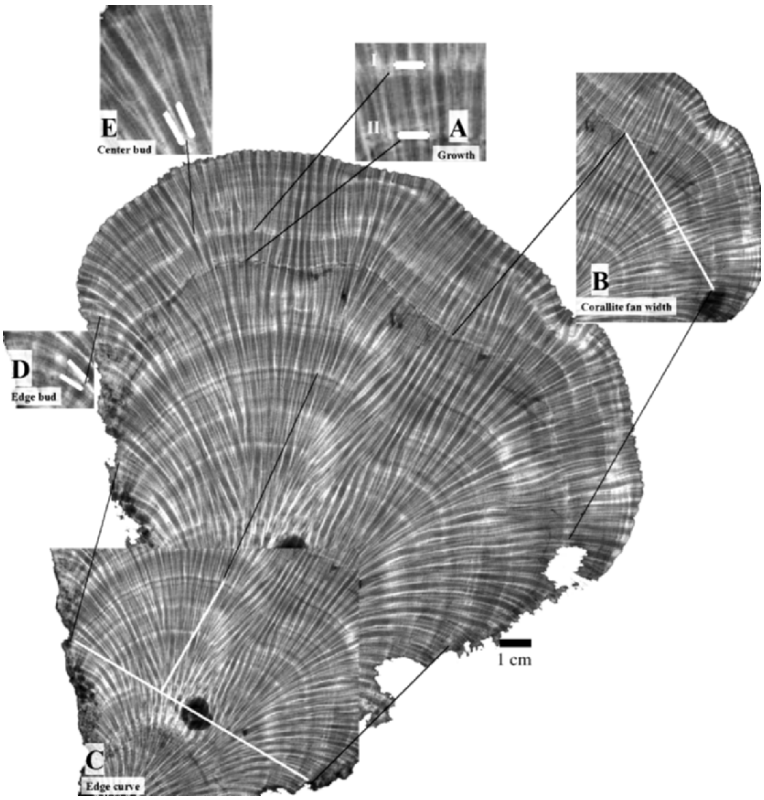

Figure 1. Longitudinal X-radiograph of a specimen of *M. annularis* from Panamá with characters described in Table 2 depicted. Characters are: (A) growth (I and II indicate high density bands), (B) corallite fan width, (C) edge curve, (D) edge bud, (E) center bud.

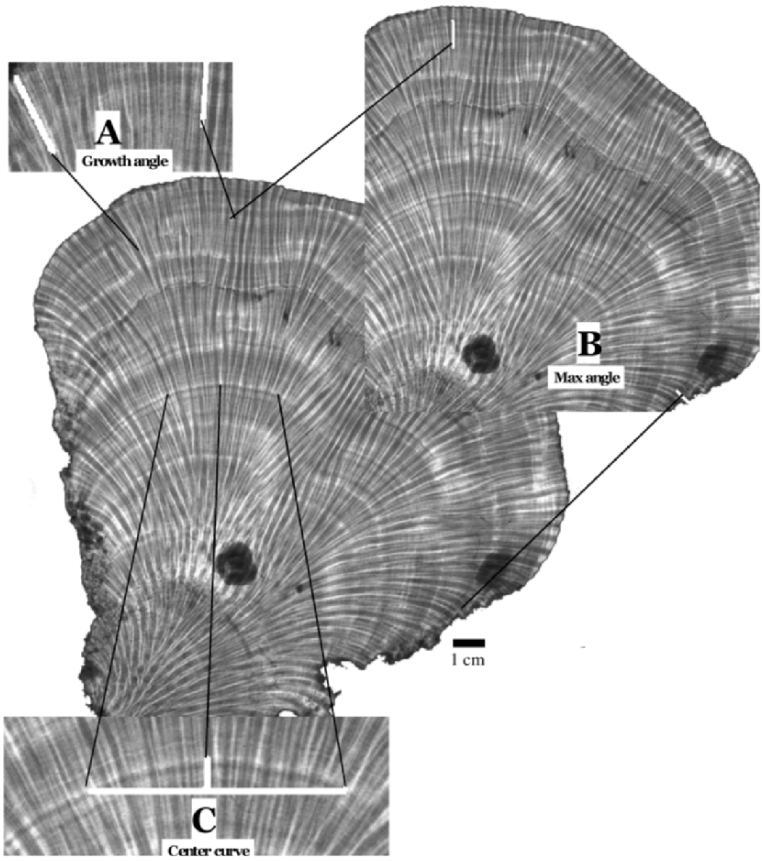

Figure 2. Depictions of characters described in Table 2 continued: (A) growth angle, (B) maximum angle, (C) center curve.

locations were evaluated using the Kruskal–Wallis test, and each species/location combination was specified as the treatment variable. Nonparametric pairwise comparisons followed procedures described by Zar (1999). Canonical discriminant function analysis (DFA) was carried out using the quadratic mode to classify specimens and identify characters important in distinguishing among species. When it was not possible to measure any given character for a specimen, the overall mean value for the character was substituted prior to running DFA.

Results

There was considerable variability in most traits both between species and locations and within a given location for a single species (Fig. 3). Despite such variability, significant differences were observed between species for all characters (Table 3). Significant differences were not observed within a species between localities. The overall means for each character are given in Table 4. Correlations between characters are shown in Table 5. For DFA, elimination of individual characters from the model revealed edge curve had little effect on canonical-scores plots and thus was eliminated from the model.

Panamá specimens were classified correctly (DFA assigned all specimens to the same species as had been determined genetically) using seven of the eight morphological characters, and the canonical-scores plots show three distinct groups, with no overlap between *M. franksi* and *M. annularis* (Fig. 5). When Belize specimens were run unclassified in the Panamá DFA, canonical-scores plots showed general agreement between visual assignments of specimens and their clustering with Panamá species (Fig. 5A).

Table 2. Characters examined, how they were measured, and the number of replicate measurements made per colony

Character	Description	Replicates
Growth (mm/year)	Growth rates were estimated based upon the distances between successive annual high density bands along the growth axis (line with maximal linear extension) (Fig. 1A).	5
Corallite-fan width (mm)	A corallite-fan system was considered to be the collection of corallites spreading from a growth axis, giving rise to a fan shaped pattern with largely convex density bands. The widths of individual corallite-fan systems were measured in each specimen by determining the distance between the two most distant points that could be identified as lying on the same high density band in a given fan (Fig. 1B).	5
Edge curve	Colony curvature was expressed as the ratio of the length of a line between the two most distant points on a single high density band and the distance (perpendicular) between that line and the highest point on the same high density band (Fig. 1C).	5
Edge bud (°)	Corallite budding angles were measured by placing lines parallel to the corallite wall of the mother and daughter polyps and calculating the angle of intersection for polyps near the edge of each corallite-fan system (Fig. 1D).	5
Center bud (°)	Same as edge bud, except polyps were near the center of the corallite fan system (Fig. 1E).	5
Growth angle (°)	The angle between the growth axis and the corallite with the greatest angle that was part of the same corallite-fan system, with the bulk of its recent growth (within two density bands) parallel to the growth axis, was measured (Fig. 2A).	1
Max angle (°)	The maximum corallite angle was determined by measuring the angle between the growth axis and a line parallel to the corallite wall of the corallite angled the furthest from the growth axis (Fig. 2B) (from Graus & Macintyre, 1982).	1
Center curve	Same as edge curve, except measurements were along the growth axis (Fig. 2C).	5

In the DFA of Panamá specimens with Barbados specimens unclassified, canonical-scores plots show the two Pleistocene forms to be distinct from each other, and neither form appears to overlap well with any single extant species (Fig. 5B).

Discussion

Characters

Many of the quantitative differences between species are consistent with both the apparent differences observed in X-radiographs and the reported morphological differences between species observed in the field. Patterns of average growth-rate variation between the members of the *M. annularis* species complex (Table 4) are consistent with those reported in other studies (Knowlton et al., 1992; Szmant et al., 1997; Pandolfi et al., 2002). The larger and more variable budding angles observed in *M. franksi* (Table 4) would seem consistent with the uneven calice distribution and greater spacing reported for *M. franksi* (Budd, 1993; Van Veghel & Bak, 1993; Szmant et al., 1997). Specimens of *M. annularis* tended to consist of single corallite fan systems, or multiple, poorly separated, corallite-fan systems arising from a single corallite fan. Such single fans were associated with smooth hemispherical and columnar growths; corallites grew at larger angles relative to the growth axis, thus resulting in higher values for maximum angle and edge curve for *M. annularis* relative to other species (Table 4; Fig. 4). In *M. faveolata*, specimens consisted of large relatively smooth regions interspersed with well-defined corallite-fan systems, likely responsible for the 'keeled' appearance of colonies. Corallite-fan systems in *M. faveolata* tended to be highly curved near their centers and gradually merged with the surrounding flatter skeleton, thus yielding relatively narrow fan systems with a high degree of center curvature (Table 4). *M. franksi*, like *M. faveolata*, tended to have relatively smooth regions, but these were frequently interrupted with

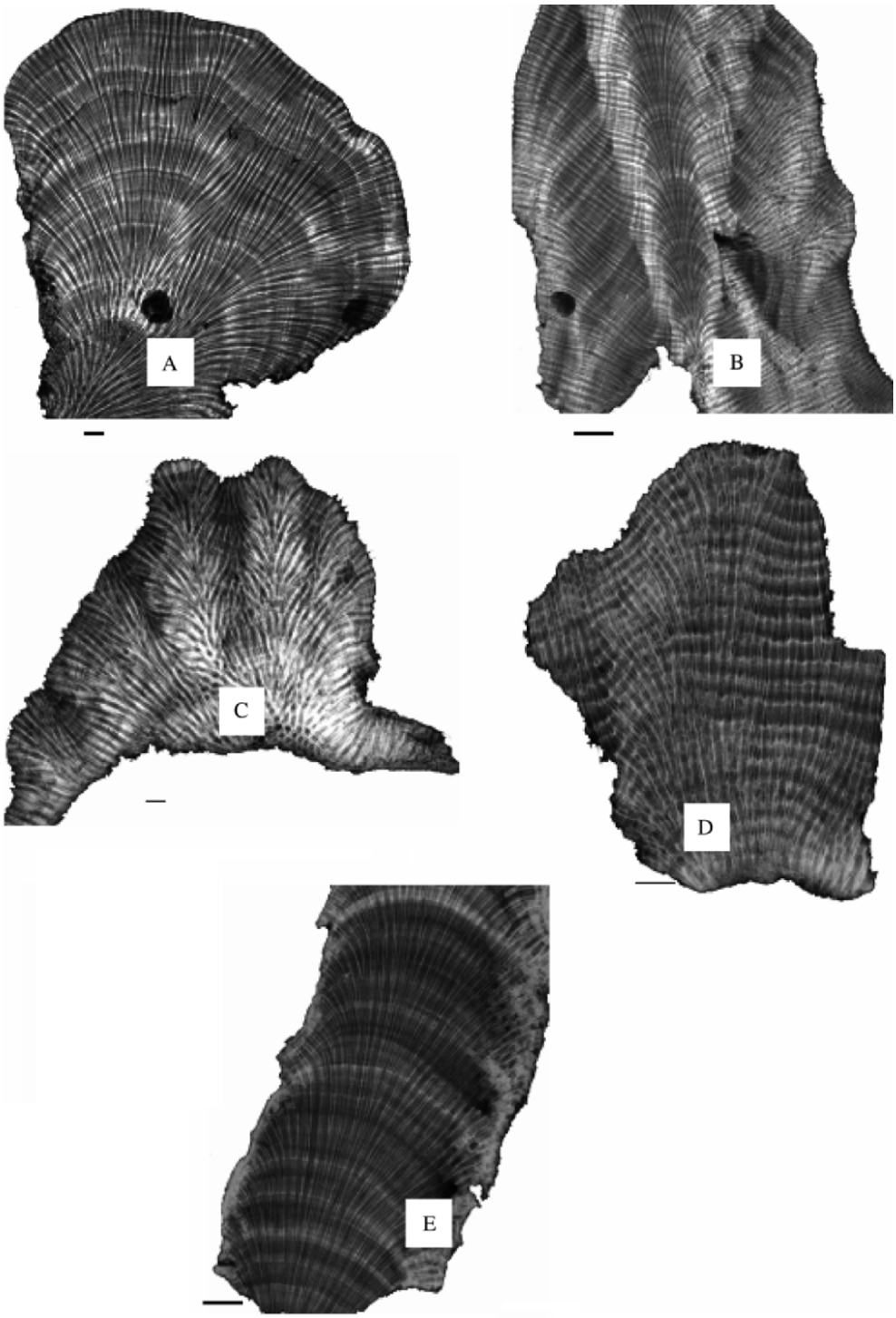

Figure 3. Longitudinal X-radiographs of modern and Pleistocene lineages of the *Montastraea annularis* species complex: (A) *M. annularis*, (B) *M. faveolata*, (C) *M. franksi*, (D) columnar, (E) organ pipe. Bars represent approximately 1 cm.

Table 3. Significant differences in each character between species at a given location as indicated by pairwise comparisons

	Location		
Character	Panamá	Belize	Barbados
Growth	A > > > K; F > > K	A > > K	OP > > > C
Corallite-fan width	A > > F; A > > K	n.s.	n.s.
Edge curve	n.s.	A > K	n.s.
Edge bud	K > > > A	n.s.	C > > OP
Center bud	K > > > A	n.s.	n.s.
Growth angle	n.s.	n.s.	OP > > > C
Maximum angle	n.s.	n.s.	OP > C
Center curve	n.s.	n.s.	OP > > C

Overall Kruskal–Wallis tests were all significant at the $p < 0.001$ level. Significance values are as follows: n.s. = not significant = $p > 0.05$; > = $p < 0.05$; >> = $p < 0.01$; >>> = $p < 0.001$. A, *M. annularis*; F, *M. faveolata*; K, *M. franksi*; C, columnar; OP, organ pipe.

small fan systems (giving rise to 'bumps'). Regions with well defined growth banding or a single extensive growth axis were rare in *M. franksi*, budding angles tended to be larger, and growth slower than in other species (Table 3). The columnar growth form consisted of very flat, linear growth bands, occasionally interrupted by small corallite-fan systems (Table 4). The organ-pipe form consisted of single corallite-fan systems with well-defined growth banding, relatively narrow widths, and more curvature than columnar forms (Table 4).

The negative correlation of corallite-budding angles with both growth and fan width (Table 5) seems consistent with the expectation that colonies exhibiting more vertical growth would tend to have corallites more or less parallel to each other; similarly, more corallites growing parallel to each other should give a wider corallite-fan system. However, corallite-budding angles showed no correlation with any of the measures of colony curvature (edge curve, growth angle, maximum angle, and center curve) (Table 5), suggesting that the angle at which polyps divide does not necessarily reflect the overall curvature of the colony. Most measures of colony curvature (edge curve, growth angle, maximum angle, and center curve) were correlated with each other as might be expected (Table 5). Edge bud and center bud were highly correlated with each other, suggesting that budding angles remain similar across the colony (Table 5).

Species differences

The clear separation of species observed in the Panamanian specimens (Fig. 5) combined with their 100% classification success demonstrates that characters based on X-radiographs effectively distinguish members of the *Montastraea annularis* species complex. As all of the characters are based on internal skeletal structures, they should be diagnostic regardless of surface weathering. Three of the most heavily weighted characters in the DFA (growth, center bud, and center curve) are readily measurable on even small skeletal sections such as might be obtained in core samples. Thus, since members of the *M. annularis* species complex differ in their stable isotope ratios (Knowlton et al., 1992), and stable isotope ratios are routinely used in paleoclimactic reconstructions (Knowlton et al., 1992; Druffel, 1997), such characters as growth, center bud, and center curve may prove valuable in differentiating among species for paleoclimactic studies.

The canonical-score plot of the Belize specimens based upon Panamanian specimens was in good agreement with visual identifications based on X-radiographs (Fig. 5A). Thus, at least over a few degrees change in latitude, X-radiographic characteristics appear to be useful in assigning identities to corals from different localities.

Barbados and Panamá specimens run together show clear groupings of the organ-pipe and columnar growth forms (Fig. 5B) described by

Table 4. Mean values for each character (sites pooled) with standard deviations in parentheses and the number of colonies measured in brackets

Species/growth form	Character							
	Growth (mm/year)	Corallite fan width (mm)	Edge curve	Edge bud (°)	Center bud (°)	Growth angle (°)	Maximum angle (°)	Center curve
M. annularis	10.1 (2.6) [29]	109 (50) [29]	0.27 (0.10) [28]	10 (2) [29]	8 (2) [29]	36 (19) [29]	120 (27) [29]	0.11 (0.05) [29]
M. faveolata	8.6 (2.5) [14]	44 (11) [14]	0.25 (0.14) [12]	13 (2) [13]	11 (2) [14]	47 (19) [14]	95 (14) [14]	0.19 (0.09) [14]
M. franksi	4.1 (2.1) [31]	62 (41) [30]	0.16 (0.06) [8]	19 (5) [18]	17 (5) [31]	29 (17) [31]	84 (27) [31]	0.09 (0.07) [31]
Columnar	5.4 (1.3) [27]	35 (15) [6]	0.16 (0.06) [12]	13 (3) [14]	9 (3) [27]	22 (17) [22]	35 (24) [27]	0.08 (0.07) [27]
Organ pipe	12.6 (2.1) [30]	66 (27) [16]	0.21 (0.08) [30]	9 (2) [30]	8 (1) [30]	50 (10) [30]	75 (12) [30]	0.11 (0.04) [30]
Overall	8.1 (3.9) [131]	73 (46) [95]	0.22 (0.10) [90]	12 (4) [104]	11 (5) [131]	36 (19) [126]	81 (36) [131]	0.11 (0.07) [131]

Table 5. Correlations between characters

	Growth (mm/year)	Corallite fan width (mm)	Edge curve	Edge bud (°)	Center bud (°)	Growth angle (°)	Max angle (°)
Corallite fan width (mm)	n.s. (0.194) [95]						
Edge curve	n.s. (0.090) [90]	n.s. (0.196) [70]					
Edge bud (°)	0.000 (−0.568) [104]	0.000 (−0.402) [82]	n.s. (−0.008) [83]				
Center bud (°)	0.000 (−0.560) [131]	0.001 (−0.332) [95]	n.s. (−0.090) [90]	0.000 (0.718) [104]			
Growth angle (°)	0.000 (0.567) [126]	n.s. (0.010) [95]	n.s. (−0.007) [89]	n.s. (−0.105) [104]	0.01 (−0.229) [126]		
Maximum angle (°)	0.002 (0.272) [131]	0.024 (0.232) [95]	0.000 (0.556) [90]	n.s. (−0.027) [104]	n.s. (0.024) [131]	0.003 (0.261) [126]	
Center curve	0.005 (0.244) [131]	0.003 (−0.301) [95]	0.000 (0.484) [90]	n.s. (0.066) [104]	n.s. (0.005) [131]	0.000 (0.361) [126]	0.001 (0.288) [131]

The significance of each correlation is shown, in parenthesis is the correlation coefficient (r), and in brackets is the number of specimens on which the correlation is based. n.s. = not significant = $p > 0.05$.

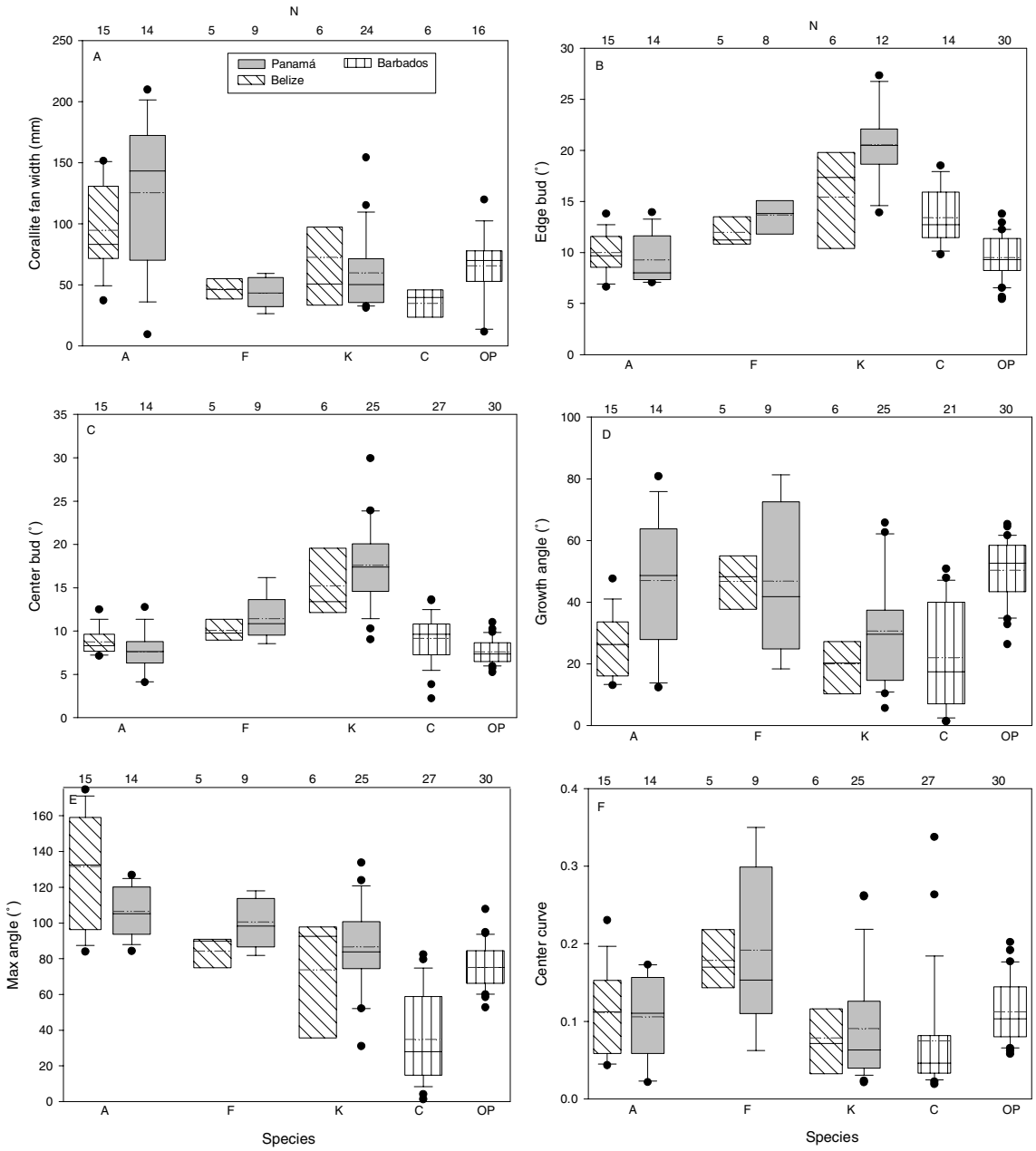

Figure 4. Box plots for selected characters. Plots show the median as a solid line, mean as a dashed gray line, quartiles as ends of boxes, extremes as whiskers, and outliers as dots. The upper horizontal axis (*N*) gives the number of specimens represented by each plot, the lower horizontal axis (Species) gives the species or growth form designation (A. *M*. *annularis*; F. *M*. *faveolata*; K. *M*. *franksi;* C. columnar; OP. organ pipe). The *Y* axis is the character represented in the plots: (A) corallite-fan width, (B) edge bud, (C) center bud, (D) growth angle, (E) max angle, (F) center curve.

Pandolfi et al. (2002). Such distinct groupings appear consistent with the separation of the organ-pipe growth form as a separate species; however, the columnar form appears to be distinct from *M*. *annularis* as well (also observed by Pandolfi et al., 2002).

Despite the apparent utility of morphological characters derived from X-radiographs in differ-

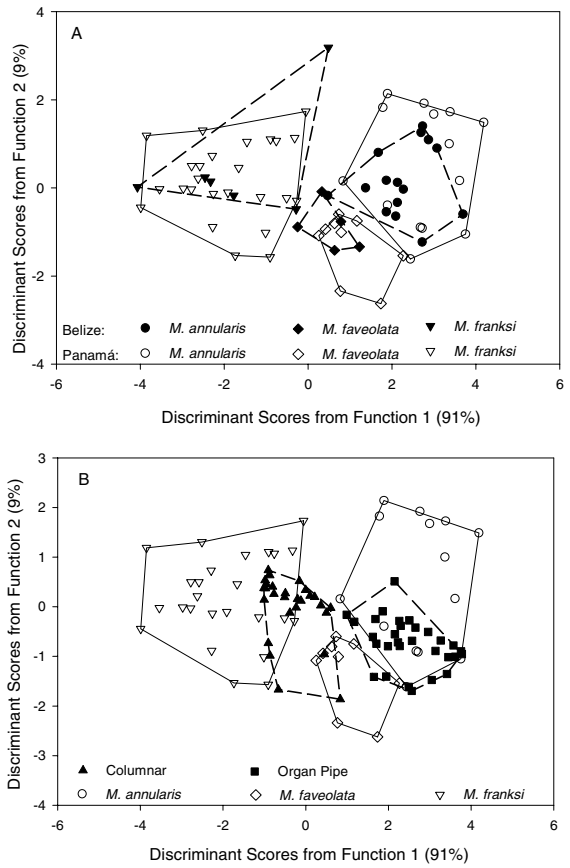

Figure 5. Plot of discriminant scores for Panamá species, based on all factors excluding edge curve. Heavily weighted characters were edge bud (−0.600), center bud (−0.572), and growth (0.547) in function 1, and fan width (0.686) and center curve (−0.454) in function 2. Variance explained by each function is given in parentheses. Symbols represent individual specimens. (A) Belize specimens were run with no identity assigned. (B) Barbados specimens were run with no identity assigned. Indicated species/form designations are based on visual assignments, not DFA assignments.

entiating species in the *M. annularis* species complex, a number of potential concerns remain to be addressed. Many morphological characters, including growth and maximum angle, have been shown to vary with light (or depth) and other environmental factors such as water energy and sedimentation (Dustan, 1975; Graus & Macintyre, 1982; Foster, 1985; Hubbard & Scaturo, 1985; Huston, 1985; Budd, 1993; Darke & Barnes, 1993). Work by Graus & Macintyre (1982) demonstrated that intercorallite spacings (likely similar to budding angles in the current study), maximum angle (determined via a different technique from that used in the current study), and growth (equivalent to the current study) are all significantly correlated with depth. Colony form changes with depth, progressing from hemispheres at shallow depths to flared columns at intermediate depths and, finally, plates at the greatest depths (Graus & Macintyre, 1982). Species composition also changes with depth, with *M. annularis* being most common at shallow depths, and *M. franksi* more abundant at greater depths (Van Veghel & Bak, 1993; Weil & Knowlton, 1994; Szmant et al., 1997). Increases in intercorallite spacings, decreasing growth, and decreasing maximum angle were all consistent with models of responses to decreasing light intensity with increasing depth (Graus & Macintyre, 1982). Changing species composition with depth would also yield a similar pattern of changes in these characters, thus the differing skeletal morphologies may represent specific adaptations by members of the *M. annularis* complex to different light regimes. The relative importance of species identity versus light availability in determining these characters, or the additional characters measured in the current study, is not known; it remains to be established whether species or environmental differences are of primary importance in determining values for a given character.

Further, caution must be taken in interpreting characters from X-radiographs since work by Darke & Barnes (1993) on *Porites* spp. demonstrates that X-radiographs present an average image and do not necessarily reflect the true positions of corallites. In *M. annularis*, the direction of corallite growth may depart from that of the apparent growth axis. This is particularly common in *M. franksi* where corallites, which are only a short distance below the surface, may have orientations approaching perpendicular to that of the apparent growth axis. Difficulties such as these may limit both the resolution of separations based on X-radiographs and the utility of radiographs for understanding internal skeletal structure. Finer resolution might be obtained through 3-dimensional imaging such as that obtained in CAT scans (Dodge, 1980).

In summary, morphological characters derived from X-radiographs appear to provide a useful means of differentiating the members of the *M. annularis* species complex. These data demon-

strate that characters from X-radiographs show similar diagnostic power to that achieved with other methods of identifying members of the *M. annularis* species complex. With further work and additional characters, especially those defining and quantifying corallite-fan systems, characteristics from X-radiographs may prove to be a valuable tool for determining species identities in both extant and extinct corals. This tool would aid identification of species when the growth form was unknown, for example from cores. Future work should address whether or not characters measured from X-radiographs are diagnostic across broad geographic areas, large intervals of time, and wide ranging environmental conditions.

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Redescription of *Bedotella armata* from Biscay Bay, northern Spain, the type species of *Bedotella* Stechow, 1913 (Cnidaria, Hydrozoa, Lafoeidae), with comments on its taxonomic position

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Key words: Hydrozoa, Leptothecata, Hebellinae, systematics, hydroids

Abstract

The circalittoral and bathyal genus *Bedotella*, comprising a unique known species endemic to the North European Atlantic (Lusitanian Province), *Bedotella armata* (Pictet, C. & M. Bedot, 1900) is redescribed based on a specimen from the Bay of Biscay, North Spain. As the holotype of the species, originally described as *Campanularia armata*, is sterile and incomplete, the present re-description includes new data concerning its morphometry, including gonothecae, and cnidom, as well as all the literature. The diagnostic characters of *Bedotella armata* are the presence of globular nematothecae, the campanuliform hydrothecae, and flabelliform, gigantic gonothecae. We corroborate the hypotheses of affinities of *Bedotella* with hydroids of the Hebellinae, most possibly with the genus *Halisiphonia*.

Introduction

The genus *Bedotella* was proposed by Stechow (1913a, p. 137) to accommodate only one species, *Campanularia armata* Pictet & Bedot, 1900, following an opinion that it was originally regarded as different from other members of the Campanulariidae, and belongs to an undescribed genus due to the presence of globular nematothecae on the stem and branches. Although accepting this species within the genus *Campanularia* Lamarck, 1816, Billard (1906a) was the first to notice its affinities with the Lafoeidae, comparing it to *Zygophylax* Quelch, 1885. Stechow (1913a), besides creating the new genus, referred it to the Hebellinae of the Lafoeidae. The trophosome of *B. armata* is, indeed, somewhat similar to that of the Hebellinae regarding the shape of the hydrothecae and also the typical stolonal habit occasionally present in some colonies. The erect portions of *Bedotella* are composed of a number of adnate tubes (denominated 'rhizocaulomic' colonies by Ramil & Vervoort, 1992), therefore a product of stolonal growth. The gonothecae are morphologically similar to those of *Halisiphonia* Allman, 1888 (cf. Ramil & Vervoort, 1992), and were first represented by Altuna (1994a) and first formally described by Álvarez-Claudio (1995), corroborating its affinities with the Hebellinae. Although the gonosome was still unknown at that time, Bouillon (1985, p. 155), in his diagnoses of the genus, said that 'les gonothèques naissent isolément'.

The goal of this study is to describe a fertile colony of *B. armata*, adding comprehensive data to the original description. We try to clarify the taxonomy of this poorly known lafoeid genus, including complementary data of morphometry and morphology (e.g. cnidom) in the description.

Material and methods

The material, three colony fragments ca. 2, 15, and 17 mm high, with a single gonotheca, was obtained in a survey of bottom communities in September 1985 at 100 m depth, 300 m NW of the 'Gaviota' offshore gas platform off the village of Bermeo (Basque Country, Spain) undertaken by the AZTI-SIO (Basque Government Fisheries Department), and was included in the unpublished PhD thesis of Altuna (1994b). The material was examined, measured, and micrographs were taken. The cnidom terminology follows Weill (1934) and Mariscal (1974); measurements of nematocysts were made on non-discharged capsules. Other general methods of study applied to Lafoeidae are from Peña & García Carrascosa (1993) and Peña Cantero et al. (1998). The material is deposited in the Museo Nacional de Ciencias Naturales, Madrid (MNCN 2.03/314, alcohol preserved).

Genus *Bedotella* Stechow, 1913a

Bedotella Stechow, 1913a, p. 137; Bouillon, 1985, p. 155.

Type species. – *Bedotella armata* (Pictet & Bedot, 1900), as *Campanularia armata* Pictet & Bedot, 1900, by monotypy (Stechow, 1913a).

Diagnosis – Colonies stolonal or erect. Erect stems rhizocaulomic, polysiphonic, without an axial tube. Hydrothecae campanulate, on pedicels of varied length, irregularly arising singly from hydrorhiza and hydrocauli; demarcated from pedicel basally by a thin hydrothecal diaphragm. Operculum absent. Nematothecae, pedicellated, globular, scattered over hydrorhiza and stem tubes.

Gonothecae solitary, flabelliform, pedicellated, arising on rhizocaulomic branches.

Remarks – When describing *Campanularia armata*, Pictet & Bedot (1900, p. 9) suggested the need for creating a new genus. They assigned the presence of nematophores represented by globular nematothecae. These nematothecae had already been seen in families other than Plumulariidae, and were similar in shape to those found in *Oplorhiza parvula* Allman, 1877. Although indeed representing a quite peculiar species to be included in the Campanulariidae, this taxonomic treatment by Pictet & Bedot (1900) was followed by a series of authors (Billard, 1906a; Marinopoulos, 1981; Aguirrezabalaga et al., 1984). Billard (1906a), however, recognized a possible relationship of *C. armata* to species of the genus *Zygophylax* Quelch, 1885, and Aguirrezabalaga et al. (1984) considered the necessity of a new genus in which to place their unusual species of Campanulariidae.

Because of a conic hypostome, Stechow (1913a) judged that the species did not belong in the Campanulariidae so created the genus *Bedotella*, which he placed in the Lafoeidae. Stechow's solution was widely followed (e.g. Stechow, 1913b, 1919; Leloup, 1940; Rees & White, 1966; Bouillon, 1985; Aguirrezabalaga et al., 1986; Altuna & García Carrascosa, 1990; Ramil & Vervoort, 1992; Altuna, 1994a; Álvarez-Claudio, 1995; Bouillon et al., 1995).

Bedotella armata (Pictet & Bedot, 1900) Figure 1, Table 1

Campanularia armata Pictet & Bedot, 1900, p. 9–10, pl. 1, figures 3–6; Billard, 1906a, p. 171; 1906b, p. 330; Stechow, 1913a, p. 137; 1913b, p. 27; Le Danois, 1948, p. 118, 175, 236; Marinopoulos, 1981, p. 175, 176; Aguirrezabalaga et al., 1984, p. 89, 90, figure 4.

Bedotella armata: Stechow, 1913b, p. 27; Leloup, 1940, p. 9; Rees & White, 1966, p. 272, 273; Aguirrezabalaga et al., 1986, p. 138; Altuna & García Carrascosa, 1990, p. 54; Ramil & Vervoort, 1992, p. 50–52, figures 7(c) and (d), 10(a) and (c); Altuna, 1994a, p. 54, figure 2A; Álvarez-Claudio, 1995, p. 265–267, figures 1 and 2; Álvarez-Claudio & Anadón, 1995, p. 238; Bouillon et al., 1995, p. 51; Medel & López-González, 1996, p. 198.

Type locality – 'Golfe de Gascogne' (=Bay of Biscay), 134 m depth.

Type specimen – Described by Pictet & Bedot (1900) from the 'Campaign [Prince de Monaco] de 1886: Stn. 58, profondeur 134 m. Golfe de Gascogne', in the collection of the 'Musée Océanographique de Monaco'. The Monaco Museum has the policy not to loan type specimens; therefore, we did not have this type specimen available for study.

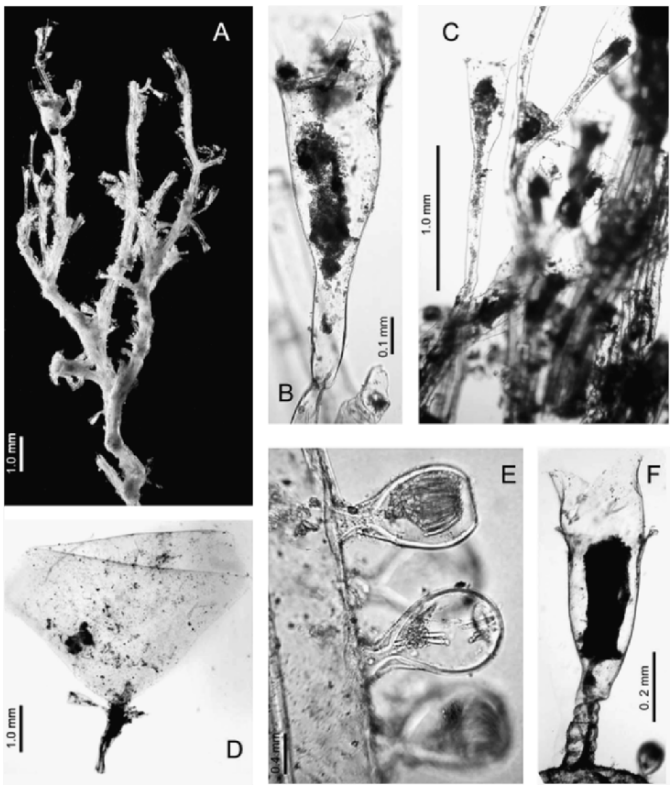

Figure 1. Bedotella armata. (A) Part of colony showing the branching pattern. (B) Rhizocaulomic hydrotheca (note the diaphragm region). (C) Detail of the colony (note long rhizocaulomic pedicel). (D) Gonotheca broken off from colony. (E) Series of nematothecae on rhizocaulus showing battery of nematocysts. (F) Stolonal hydrotheca with short, annulated pedicel and one renovation (note the hydranth constrained by diaphragm).

Description – Colony erect, polysiphonic up to its distal parts, with rhizocaulomic branches arising from any of constituent tubes. Basal part of stem ca. 0.48 mm in diameter, distal part 0.23–0.28 mm. Erect stem arising from a rootlike tuft of hydrorhizal tubes (seen in one fragment), 0.06–0.12 mm in diameter. Branches up to fourth-order. Hydrothecal pedicels arising from apical stem and rhizocaulomic branches. Primary branches irregularly arranged in several planes, up to 12 mm long, completely polysiphonic, with no branching pattern and no definite main stem, 0.26–0.46 mm in diameter. Lower-order branches and hydrothecae arranged in all planes. Polysiphonic stem and main branches not divided into internodes and without axial apophyses and septa. Lateral branches arising at angles of 30–60° in relation to main stem. Hydrothecal pedicels 0.23–0.84 mm long, 0.07–0.11 mm in diameter at base, of relatively uniform diameter, ending at hydrothecal diaphragm, with a series of 3–8 basal rings or undulations, sometimes with 2–3 distal rings or undulations just before hydrothecae. Nematothecae numerous, solitary or in rows of up to 7, on short pedicels, irregularly distributed on polysiphonic tubes and single branches; nematotheca globular with distal circular aperture, 0.09–0.11 mm in length, 0.07–0.09 mm in maximum diameter, 0.03–0.04 mm in diameter at aperture; nematothecal pedicel, 0.02–0.03 mm long, 0.02–0.03 mm in diameter. Median to apical portion of nematothecae with bundle of nematocysts parallel to nematothecal long axis.

Hydrothecae campanulate, with almost parallel walls from aperture to middle portion, gradually narrowing towards pedicel. Pedicel of varied length, some slightly asymmetric in the basal third. Hydrothecae 0.46–0.58 mm long (from diaphragm

to aperture), 0.14–0.16 mm in diameter at diaphragm level, 0.22–0.42 mm diameter at aperture, some slightly symmetric. Hydrothecal walls not rigid, with smooth and thin perisarc and unthicken parts. Diaphragm thin, apparently circular, generally transverse in relation to hydrothecal long axis; desmocytes distributed on the hydrothecal wall, above diaphragm. Hydrothecal aperture circular, perpendicular to hydrothecal long axis; rim even and sometimes strongly flared, with up to 5 hydrothecae without operculum. Hydranths tubular, 0.32–0.37 mm in height, 0.13–0.18 mm in diameter at base of tentacles, basal region constricted by diaphragm, without caecum; apical region with one whorl of ca. 12–16 tentacles, hypostome conical.

Gonotheca triangular, with thin, smooth and hyaline perisarc, vertex slightly rounded basally, truncated apically, strongly compressed, arising on rhizocaulomic branches on short pedicel (ca. 0.22 mm long and 0.21 mm wide distally), with a narrow distal slit as aperture. Gonotheca 3.90 mm in total height excluding pedicel, 5.50 mm wide at aperture, from pedicel one side 3.13 mm long, other side 3.75 mm long.

Nematocysts holotrichous isorhiza (seen discharged), 34–43 × 5–6 μm (37.80 ± 2.49 × 5.45 ± 0.44, $n = 10$), rice-shaped, abundant in nematothecae, occasionally in coenosarc and hydranth body. Nematocysts from tentacles not seen.

Additional data – Ramil & Vervoort (1992, p. 50) wrote 'stolonal colonies consist of solitary, creeping tubes (usually on other hydroids) or an anastomosing, irregular network of stolonal tubes (usually on worm-tubes). Rhizocaulomic colonies 5–7 mm high [although the authors reported colonies of 12 mm in their list of material], composed of a number of adnate stolons, basally attached by means of creeping stolons that in some colonies may bear hydro- and nematothecae (Fig. 10(a) and (b))'. Álvarez-Claudio (1995, p. 265) reported a ring of desmocytes at the base of the hydrothecae

Table 1. Morphometric data of *Bedotella armata* from the material studied herein (measurements in mm)

Characters	Mean ± SD (range) (*n*)
Diameter of hydrorhiza	0.06–0.12 (5)
Diameter of stem (base)	ca. 0.48 (1)
Diameter of pedicel (distal)	0.23–0.28 (5)
Diameter of primary branches	0.34 ± 0.09 (0.26–0.46) (5)
Length of hydrothecal pedicel	0.54 ± 0.25 (0.23–0.84) (5)
Diameter of hydrothecal pedicel	0.09 ± 0.02 (0.07–0.11) (5)
Number of pedicellar annulations (base)	3–8 (5)
Number of pedicellar annulations (apical)	0–3 (5)
Length of hydrotheca	0.51 ± 0.05 (0.46–0.58) (5)
Diameter at diaphragm	0.15 ± 0.01 (0.14–0.16) (5)
Diameter of aperture	0.28 ± 0.08 (0.22–0.42) (5)
Length of hydranth	0.35 ± 0.02 (0.32–0.37) (5)
Diameter of hydranth at tentacle base	0.15 ± 0.02 (0.13–0.18) (5)
Number of tentacles	12–16 (5)
Length of nematotheca	0.10 ± 0.01 (0.09–0.11) (10)
Diameter of nematotheca (maximum)	0.081 ± 0.007 (0.07–0.09) (10)
Diameter of nematothecal aperture	0.035 ± 0.005 (0.03–0.04) (10)
Length of nematothecal pedicel	0.025 ± 0.005 (0.02–0.03) (10)
Diameter of nematothecal pedicel	0.025 ± 0.005 (0.02–0.03) (10)
Length of gonothecal pedicel	0.22 (1)
Diameter of gonothecal pedicel	0.21 (1)
Height of gonotheca	3.90 (1)
Width of gonotheca at aperture	5.50 (1)

Table 2. Morphometric data of records of *Bedotella armata* (Pictet & Bedot, 1900)

Characters	Bay of Biscay (present study)	'*C. armata*', Bay of Biscay (Pictet & Bedot, 1900)	Atlantic, off Gibraltar Strait (Ramil & Vervoort, 1992)	Bay of Biscay (Álvarez-Claudio, 1995)
Colony height	15	10	up to 12	up to 45
Colony diameter at base	0.48	0.30		
Length of hydrothecae	**0.46**–0.58	0.50–0.60	0.47–**0.67**	
Diameter of hydrothecae at rim	**0.22**–**0.42**	0.30–0.32	0.23–0.39	
Length of pedicel	**0.23**–0.84	0.50–**2.00**	0.62–1.56	
Diameter of pedicel	**0.07**–**0.11**	0.08	0.07–0.09	
Length of nematothecae	**0.09**–0.11	**0.15**	0.10–0.11	
Maximal diameter of nematotheca	**0.07**–**0.09**	0.08	0.07–0.08	
Length of nematothecal pedicel	**0.02**–0.03		**0.04**	
Height of gonothecae	**3.90**			**2.67**–3.00
Maximal diameter of gonothecae	**5.50**			**2.73**–3.15
Height of gonothecal pedicel	**0.22**			**0.21**
Nematocyst length (in μm)	**34**–43		45–**50**	

In bold are marked the extreme values recorded for the species. All data in mm except nematocysts in μm.

and described the gonothecae with 'the surface to be wrinkled, probably as an artefact of preparation'. For morphometrical data, see Table 2.

Remarks – Ramil & Vervoort (1992; also Álvarez-Claudio, 1995) found both stolonal and erect colonies, the latter referred to as 'rhizocaulomic' colonies due to their stolonal nature. This is in agreement with the original description by Pictet & Bedot (1900, p. 10), who stated that irregular pedicels arise separately on stems or in small sets, and that the branches might be formed by sets of pedicels running together. The erect portions of the colonies could therefore be interpreted as derived from typical stolonal growth, which is congruent with the character in related genera of Hebellinae Fraser, 1912 (viz., *Hebella* Allman, 1888; *Halisiphonia* Allman, 1888; and *Scandia* Fraser, 1912) (see Calder, 1991). *Filellum* Hincks, 1868, the other Lafoeidae genus with stolonal colonies, is included in the Lafoeidae A. Agassiz, 1865, due to the absence of a diaphragm. It is noteworthy that the polysiphonic habit of *B. armata*, in which there is no main axis, with branches arising from any tube of the fasciculation, is not equivalent to those found in other genera of Lafoeidae, both in the Lafoeinae (viz., *Acryptolaria* Norman, 1875; *Cryptolarella* Stechow, 1913; *Grammaria* Stimpson, 1854) and Zygophylacinae Quelch, 1885 (viz., *Zygophylax* Quelch, 1885; *Abietinella* Levinsen, 1913; *Cryptolaria* Busk, 1857), in which the branches arise from a main axis (cf. Allman, 1888). Anyhow, the plasticity of the colonies of lafoeids, from stolonal to erect habits, even on the ontogeny of the same species, is well-known (cf. e.g. Vervoort, 1966).

The gonotheca of *B. armata* is also similar to those of the Hebellinae, being solitary, not set into a coppinia, a structure typical of the Lafoeinae and Zygophylacinae (the genus *Cryptolarella* seems to be an exception among these subfamilies). However, the nature of the gonophore in *Bedotella* is unknown, whether it is a free medusa/medusoid or fixed sporosac. The Hebellinae includes both free medusae and eumedusoids with gametes on radial canals (*Hebella*) and fixed sporosacs (*Scandia*) (cf. Boero et al., 1997a), but much has still to be discovered about the reproduction of its other genera.

Pictet & Bedot (1900) misinterpreted the presence of oocytes in their infertile material. The structure of the gonophores was not known until recently. Fertile colonies were quoted by Aguirrezabalaga et al. (1986) and no detailed description was given. Ramil & Vervoort (1992, p. 52) reported gonothecae to be described in material from Bay of Biscay (northeast Atlantic) collected by Álvarez-Claudio, and remarked that the gonothecae would have 'affinities with that met in the genus *Halisiphonia* Allman, 1888'. Although the gonotheca was firstly described by Álvarez-Claudio (1995), it was figured earlier by Altuna (1994a, fig. 2(A)). The gonotheca of *B. armata* is flabelliform, almost triangular, arising from the rhizocaulus (referred as 'branches' in Álvarez-Claudio, 1995 fig. 1(b), here interpreted as the 'rhizocaulomic' arrangement proposed by Ramil & Vervoort, 1992). Similar gonothecae are seen in species of *Halisiphonia* (cf. Vervoort, 1966; Ramil & Vervoort, 1992; Álvarez-Claudio, 1995), especially concerning the shape, viz., *H. megalotheca* Allman, 1888, *H. galatheae* Kramp, 1956, and *H. arctica* Kramp, 1932. *Halisiphonia spongicola* Haeckel, 1889 (known only from the original description, with type material lost), has gonotheca 'not compressed or spatuliform, with a slit-shaped opening; their transverse section and the distal opening are circular' (Haeckel, 1889, p. 77, pl. 4, fig. 9). Finally, the gonophores of *H. nana* Stechow, 1921, are still unknown. The main difference between the gonothecae of *B. armata* and those of *Halisiphonia* spp. is the gigantic dimensions of the former.

In contrast, nematothecae are not present in any other Hebellinae, being, however, characteristic of other Lafoeidae Zygophylacinae, viz., *Zygophylax*, *Abietinella*, and *Cryptolaria*. The structure of their nematothecae is nonetheless distinct, having also a fixed number and position on the apophyses of the hydrothecae and/or branches. Therefore, it is quite reasonable to consider the two types of nematothecae not homologous. In addition, these genera have in common hydranths with a characteristic abcauline caecum, a character absent in *Bedotella*.

Bedotella armata is apparently endemic to the Lusitanian Province (in the sense of Briggs, 1974) of the European North Atlantic. It occurs mainly in the Bay of Biscay, with records only from the southern sector (Galicia, Asturias, and the Basque Country), where the species has the northern limit of its distribution. It was also recorded in the

Mediterranean Sea (150 m off the French coast by Marinopoulos, 1981, as *C. armata*, overlooked in the synopsis of Boero & Bouillon, 1993; see Boero et al. 1997b; Alboran Sea and Gibraltar Strait proper by Ramil & Vervoort, 1992) and Azores (Leloup, 1940).

The species is found on other species of hydroids, such as *Diphasia alata* Hincks, 1855 (Pictet & Bedot, 1900, p. 9), *Polyplumaria flabellata* G.O. Sars, 1874 (Billard, 1906a, p. 171), *Aglaophenia tubulifera* Hincks, 1861 (Ramil & Vervoort, 1992, p. 50), on other cnidarians, such as *Dendrophyllia cornigera* Lamarck, 1816 (Aguirrezabalaga et al., 1984, p. 89) and *Lophelia pertusa* Linnaeus, 1758 (Álvarez-Claudio, 1995, p. 265), and on tubes of polychaetes (Álvarez-Claudio, 1995, p. 265). Eurybathic species, it has been found from 100 to 893 m (134 and 411 m in the Bay of Biscay, after Pictet & Bedot, 1900 and Billard, 1906a, respectively; 845–880 m off the Azores Archipelago after Leloup, 1940, quoted by Rees & White, 1966; 195–580 m in the Atlantic region of the Gibraltar Strait by Ramil & Vervoort, 1992; 183–893 m in the Bay of Biscay, after Álvarez-Claudio, 1995, and Álvarez-Claudio & Anadón, 1995; and 100 m in the Bay of Biscay herein, the shallowest record for the species). Still, Le Danois (1948, p. 118, 175) quoted the species in lists of the epifauna of the continental edge and coralline facies of the North Iberian sector of the Bay of Biscay.

The species probably has a wide reproductive period in the Bay of Biscay, with gonothecae reported in April, July (Álvarez-Claudio, 1995), and September (herein).

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First full description of the large physonect siphonophore *Halistemma amphytridis* (Lesueur & Petit, 1807)

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Key words: Cnidaria, Hydrozoa, Siphonophora

Abstract

The physonect siphonophore *Halistemma amphytridis* (Lesueur & Petit, 1807), previously known as *Stephanomia amphytridis*, was originally described in part from siphosomal material only. This paper demonstrates that the siphosome of a large mature *Halistemma* colony, collected during the 'Snellius' Expedition to Indonesia, is conspecific with two siphosomal samples of the above species taken by Bigelow on the 'Albatross' Expedition to the eastern tropical Pacific. Furthermore, a gap in the literature is filled herein with the first description of a *H. amphytridis* nectosome. The nectophores are larger and more prismatic than those of other published *Halistemma* species, with two vertical-lateral ridges, an incomplete lateral ridge, lateral radial canals of the nectosac more extensively looped, and a pallial canal with short diverticulum into the mesoglea. Siphosomal zooids including gastrozooids, palpons, bracts and gonophores are redescribed. The mature tentillum (on the gastrozooid tentacle) is unicornuate, with a vestigial involucrum, a cnidoband with 4–5 coils which is more extended than in other *Halistemma* species (except *H. cupulifera* Lens & van Riemsdijk, 1908), and a terminal filament without a swollen terminal process at the tip.

Introduction

A large, well-preserved physonect siphonophore collected from the surface of the Flores Sea, Indonesia, during the Dutch 'Snellius' I Expedition is identified as a giant specimen of the agalmatid genus *Halistemma*. Since the siphosome is comparable with material of *Stephanomia amphytridis* in the Smithsonian Institution collected by Bigelow (1911), this 'Snellius' specimen is here referred to *H. amphytridis* (Lesueur & Petit, 1807). *H. amphytridis* nectophores are here described and illustrated for the first time, and siphosomal zooids are re-described.

Material and methods

Material examined includes:

The subject specimen of this paper from the Museum Naturalis, Leiden, The Netherlands, collected 22.08.1930 in the Flores Sea, Indonesia, from 7° 55.0′ S, 122° 12.5′ E (Station 317a) (Goodman, personal communication) during the 'Snellius' I Expedition 1929–1930, and preserved in formalin.

Two samples of siphosomal material from the Smithsonian Institution, Washington (USNM 28303 and 28305), collected 28.12.1904 in the eastern tropical Pacific Ocean circa 600-0 m from 15° 05′ 00″ S, 99° 19′ 00″ W (Station 4705) during the 'Albatross' Expedition 1904–1905, identified by H. B. Bigelow as *Stephanomia amphitridis*, and preserved in alcohol.

Other material registered as *Halistemma* from The Natural History Museum, London (NHM) (Reg. Nos. 1949.11.10, 1949.11.10.14-16, 1959.8.10.80-82, 1974.4.26.5, 1974.7.11.1, 1986.1.6.15) and several unregistered *Halistemma* specimens (none referable to *H. amphytridis*) together with *Halistemma rubrum* (Vogt, 1852) from the

Mediterranean Sea (Reg. Nos. 1952.9.23.285-294, 1952.9.23.297-300, 1952.9.23.304, 1952.9.23.305-311, 1953.7.2.23), *H. striata* Totton, 1954, from the Atlantic Ocean (Reg. No. 1973.4.26.5), all identified by A.K. Totton, and a paratype of *Halistemma transliratum* Pugh & Youngbluth, 1988, from the Atlantic Ocean (Reg. No. 1987.4.1.1).

Specimens were transferred either to a petri dish and examined under a Zeiss binocular microscope, or to a cavity slide and examined using an Olympus CH 30 microscope, and illustrated using an attached camera lucida. Terminology for the descriptions is taken mainly from Mapstone (2003), with ridge names from Pugh & Youngbluth (1988).

Abbreviations in the present Figures are: alr – apico-lateral ridge; alr_1 – inner branch of alr; alr_2 – outer branch of alr; alr_t – tooth on alr; anb – apical nematocyst button; axp – axial process of nectosac; axw – axial wing; ba – basigaster of gastrozooid (cnidogenic band); bc – bracteal canal; bcs – bracteal canal scar; bl – bracteal lamella; br – bract; cb – cnidoband; chr – ostial chromatophore; co – column of gastrozooids; cp – cupulate process; df – dorsal furrow; dg – dorsal groove; div – diverticulum (of pallial canal); doa – dorsal opaque area; drc – dorsal radial canal; dt – dorsal tooth; el – elastic ligaments; fgd – female gonodendron; fgo – female gonophore; gmo – gastrozooid mouth; go – gonophore; grc – gonophore radial canal; gzt – gastrozooid tentacle; hs – hepatic stripe; ib – immature bract; ig – immature gastrozooid; ilr – infra-lateral ridge; inv – involucrum; itt – immature tentillum; lf – lateral furrow; lr – lateral ridge; lrc – lateral radial canal; lrc-abl – abaxial loop of lrc; lrc-axl – axial loop of lrc; lrc-dl – dorsal loop of lrc; lrc-ll – lateral loop of lrc; lrc-ol – ostial loop of lrc; lrc-vl – ventral loop of lrc; mgo – male gonophore; mp – mouthplate; n – nectophore; nb – nectophore bud; nbz – nectosomal budding zone; nem – nematocyst; nml – nectophoral muscular lamellae; no – notch; ns – nectosac; nst – nectosomal stem; odf – ostio-dorsal furrow; olf – ostio-lateral furrow; orc – ostial ring canal; ot – ostium; pal – palpon; pal_1, pal_2 – palpons identified in Figure 3j; pas – pallial canal scar; pcl – palpacle; pcl_1 – palpacle of pal_1; pdv – proximal diverticulum (of bracteal canal); pe – pedicel of tentillum; pec – pedicular canal; peg – pedicel of female gonodendron; pem – pedicel of male gonophore; pn – pneumatophore; pnd - pneumadenia (gas gland); pns – pneumatosaccus; pp – proximal point (of bract); sbz – siphosomal budding zone; sst – siphosomal stem; t – tooth; tb – thrust block; tf – terminal filament; tr – tentacle rudiment; tt – tentillum; ve – velum; vf – ventral furrow; vk – ventral keel; vlf – vertical-lateral furrow; $vlr_{1\text{-}2}$ – vertical lateral ridges 1 and 2; v – ventral ridge; vrc – ventral radial canal.

Taxonomic account: Halistemma amphytridis *(Lesueur & Petit, 1807), Figures 1–3*

Stephanomia amphytridis Lesueur & Petit, 1807: pl. 29, figure 5 (description of figure 5 in Péron, 1807, p. 45); Totton, 1954: 46.
? Stephanomia foliacea Quoy & Gaimard, 1833: 74, pl. 13, figures 8–12.
Stephanomia amphitridis Huxley, 1859: 72, pl. 6; Schneider, 1898: 118; Bigelow, 1911: 287, pl. 18, figures 1–8; Kawamura, 1953: 110.
Stephanomia amphitritis Agassiz, 1862: 368.
? *Phyllophysa foliacea* Agassiz, 1862: 369.
? *Stephanomia nereidum* Haeckel, 1888a: 40; 1888b: 221.
Phyllophysa squamacea Haeckel, 1888a: 40; 1888b: 225.
Cupulita amphitrites Bedot, 1896: 408.
? *Stephanomia* sp. Lens & van Riemsdijk, 1908: 84, pl.15, figures 113–114.
Halistemma amphytridis Totton, 1965: 60; Bonnemains & Carré, 1991: 54, figures 1 and 12; (non Daniel, 1974: 47, figure 3h–j; Daniel, 1985: 75, figure 16a, b; Mackie et al., 1987: 117).

Diagnosis

Halistemmid with prismatic dorso-ventrally flattened nectophores having two complete vertical lateral ridges and incomplete lateral ridge; lateral radial canals with six loops; bract with distal tip of canal terminating in small circular apical nematocyst swelling; terminal process of unicornuate tentillum with small cupulate process.

Description of 'Snellius' specimen

A schematic representation of the whole colony of *Halistemma amphytridis* is illustrated in Figure 1a.

Pneumatophore

3 mm × 1.25 mm, with numerous endodermal septa enclosing upper gas cavity (pneumatosaccus), with red pigmented apex and lower gas gland (pneumadenia) (Fig. 2b).

Nectosome

Nectosomal stem orange, much contracted at 31 mm long, 3.5 mm diameter (Fig. 2b); ventral nectosomal budding zone with numerous buds and several immature attached nectophores, largest with two prominent swellings, probably chromatophores; main stem with dorsal groove and 43 identifiable contracted nectophoral muscular lamellae in ventral mid-line.

Nectophores

Forty one detached matures and two detached immatures. Matures typically 20 mm × 22 mm length by width (upper view) (maximum length 23 mm), 9 mm height (lateral view), with paired ridges and wedge-shaped axial wings; mouthplate with small median notch, protruding < 2 mm from nectosac (Fig. 1b and c). Apico-lateral ridge 'alr' delimits dorsal (upper) and lateral surfaces, rises to high point marked by small tooth 'alr_t', and divides at ostium into inner branch alr_1 and outer branch alr_2 (Figs 1b, d and 2d); infra-lateral ridge 'ilr' delimits ventral (lower) and lateral surfaces and merges imperceptibly with edge of mouthplate ostially (Figs 1c, d and 2d); two

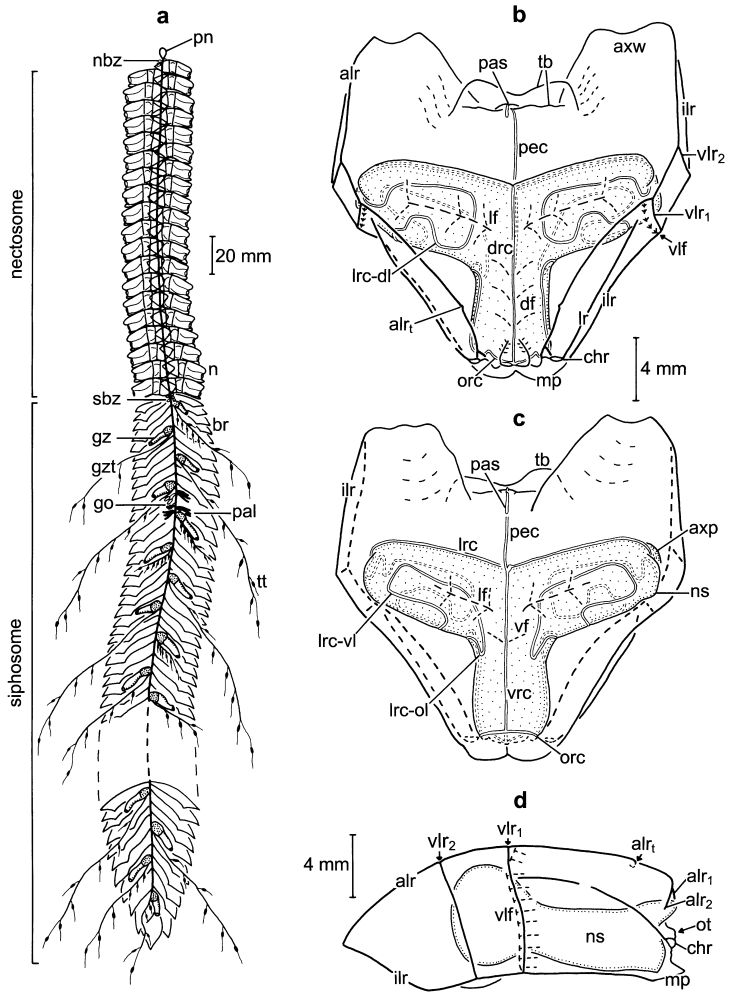

Figure 1. Halistemma amphytridis. (a) Schematic representation of whole colony with gonophores and palpons shown in one cormidium only. (b and c) Upper and lower views of mature nectophore. (d) Lateral view of mature nectophore to show ridges. Abbreviations listed under section Materials and methods'.

complete vertical-lateral ridges 'vlr_1, vlr_2' connect apico-lateral and infra-lateral ridges (Figs 1b and d); lateral ridge 'lr' extending from vertical lateral furrow to swollen ostial chromatophore plaque, course parallel to apico-lateral ridge (Figs 1a, d and 2d); short ventral ridge from junction of apico-lateral and infra-lateral ridge along edge of wedge-shaped axial wing (Fig. 2c).

Surface with furrows including dorsal furrow in dorsal mid-line from ostium, dividing at mid-nectophore height into two diagonal lateral furrows (Figs 1b and 2d); short ventral furrow in ventral mid-line arises some distance from ostium and divides into two short lateral furrows (Fig. 1c); thin opaque line from notch of mouth-plate to base of ventral furrow (not illustrated); prominent vertical lateral furrow on each lateral surface, adjacent to (and ostial of) vlr_1 ridge (Figs 1b, d and 2a); two short ostio-dorsal furrows above ostium on each side of dorsal opaque area (Fig. 2d); two small ostio-lateral furrows on lateral sides of ostium separate chromatophore swelling from alr_2 (Fig. 2d).

Thrust block between axial wings broad, bi-lobed in upper and lower views, typically shorter than wings (Fig. 1b and c), atypically longer, and undercut axially; pallial canal in mid-line on axial surface with short blind-ending diverticulum into mesoglea from upper end (Fig. 2e), connecting via long pedicular canal to radial canals of nectosac (Figs 1a, b, 2c and e). Nectosac T-shaped with two broad axial processes (Fig. 1b and c) and thick musculature; radial canals thin, arise together from pedicular canal, with dorsal and ventral straight, laterals each straight to axial nectosac process, then with six loops and short straight

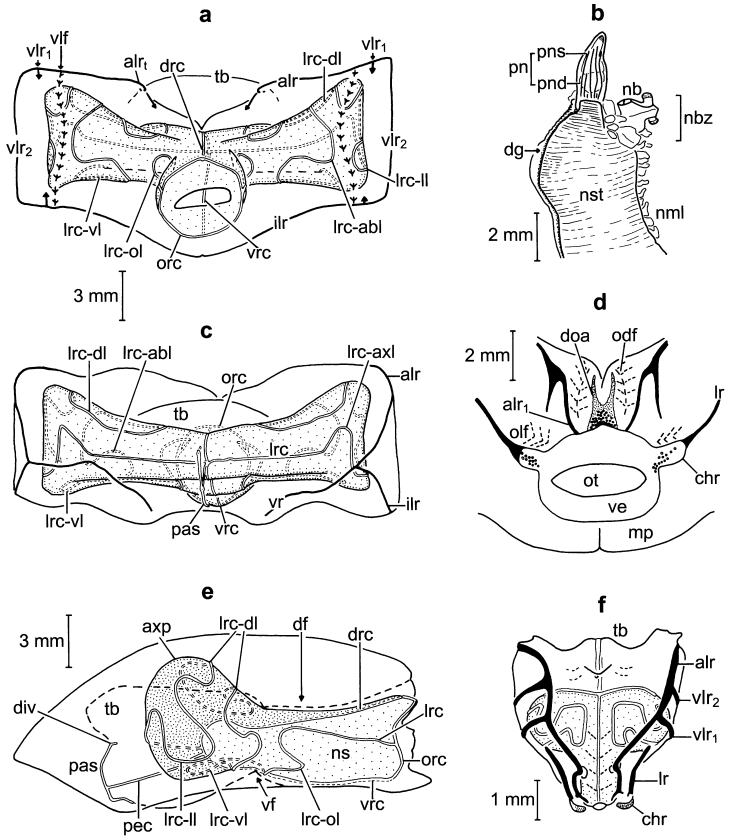

Figure 2. Halistemma amphytridis. (a and c). Abaxial and axial views of mature nectophore to illustrate the course of lateral, radial, and other canals (some ridges omitted from a). (b) Apex of nectosome, lateral view. (d) Detail of ostial region of mature nectophore. (e) Lateral view of nectophore to illustrate the course of radial canals. (f) Upper view of immature nectophore. Abbreviations listed under Materials and methods.

section to ostial ring canal (Figs 1b, c, 2a, c and e); loops include small upward axial loop 'lrc-axl' on axial process of nectosac (Fig. 2c), larger downward loop 'lrc-ll' on flattened lateral end of axial process (Fig. 2e), extensive dorsal loop 'lrc-dl' on dorsal nectosac surface (Figs. 1b, 2a and c), small abaxial loop 'lrc-abl' on abaxial nectosac surface (Fig. 2a), extensive ventral loop 'lrc-vl' on ventral nectosac surface (Figs 1c, 2b, c and e), and final small upward ostial loop 'lrc-ol' on lateral nectosac surface (Fig. 2e); ostial ring canal wavy in lateral view, follows contours of ostial end of nectosac (Figs 1b and 2e).

Immature nectophores 4.5–4 mm × 14.5–17.5 mm length by width; ridges prominent, axial wings short and level with thrust block (Fig. 2f); furrows absent in smallest nectophore, vertical lateral furrow deep in larger immature; apico-lateral ridges deviate around tooth alr_t before reaching ostium; ostial chromatophores prominent.

Siphosome

Siphosomal stem orange, much contracted, including one length contiguous with nectosome

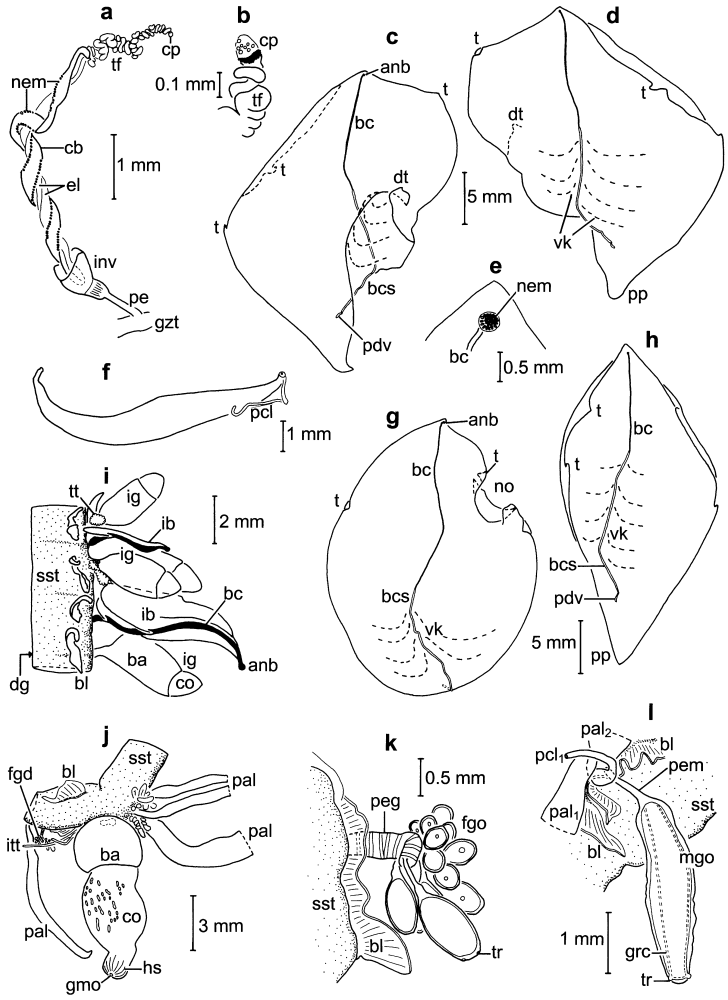

Figure 3. Halistemma amphytridis. (a) Mature tentillum. (b) Detail of cupulate process. (c and d) Upper and lower views of lateral bract with dorsal concavity. (e) Detail of tip of mature bract to show apical nematocyst pit. (f) Palpon. (g) Lower view of ventral bract. (h) Lower view of dorsal bract. (i) Lateral view of siphosomal stem below budding zone to show attached siphosomal elements. (j) Gastrozooid attached to stem. (k) Gonodendron. (l) One attached male Gonophore. Abbreviations listed under section Materials and methods'.

having short budding zone at apex, and four separate lengths, with total contracted length of approximately 155 mm; three contiguous cormidia on one fragment, 4–6 mm in length, marked by three contiguous gastrozooids.

Bracts

217 loose bracts and many small attached immature bracts, translucent with mesoglea thicker proximally than distally; mature bracts typically flaccid, immature bracts turgid; three types identified including 161 detached 'dorsal' bracts, 44 'lateral' bracts, and 12 'ventral' bracts; small number with characters intermediate between dorsal and lateral types; average length of matures 24–26 mm.

Dorsals: 88 matures, up to 32 mm × 19 mm length by width, leaf-shaped with acute angle to pointed distal tip and with proximal point; typically two pairs of approximately symmetrical lateral teeth (Fig. 3h), atypically with staggered teeth; lower surface with downward projecting ventral keel in proximal part, typically flanked by two shallow concavities, and with elongate bracteal canal scar; origin of scar slightly offset from midline, being closest to lateral surface flanking stem in life; scar with short diverticulum penetrating into mesoglea from proximal end; bracteal canal thin, typically straight, close to lower (ventral) surface, deviating through mesoglea to upper (dorsal) surface apically and terminating as small swollen apical button in cup-shaped depression filled with nematocysts (Fig. 3e); upper surface typically flat, atypically with weak mid-dorsal ridge extending short distance proximally from apical nematocyst button. Fifteen immatures <27 mm × 17 mm length by width; with teeth either present and typically symmetrical, or represented by swellings only; thick prominent bracteal canal with prominent apical nematocyst button (Fig. 3i), thick scar and very short proximal diverticulum, origin of scar somewhat asymmetric.

Laterals: 31 matures, up to 31 mm × 20 mm length by width, typically concave, markedly asymmetric and probably attached to stem in enantiomorphic (mirror-image) pairs, but specific pairs not identifiable in present material; obtuse angle to distal tip (marked by apical nematocyst button) and with proximal point; ventral keel displaced either to right or left and flanked by concavities (Fig. 3d); teeth asymmetric, typically two on outer lateral border (furthest from stem), and 1–2 on inner border; in bracts with only one lateral tooth on stem side, a second tooth is displaced onto upper surface adjacent to deep dorsal concavity (Fig. 3c); in bracts with two teeth on stem side, teeth closer to apex than those on outer lateral margin and upper surface smooth (not illustrated); ventral keel with elongate bracteal canal scar and short proximal diverticulum into mesoglea; bracteal canal thin with apical nematocyst button. Fifteen immatures <23 mm × 14 mm length by width, markedly asymmetric, with thick bracteal canal, typically large apical button; other variations as for mature bracts.

Ventrals: 10 matures, up to 27 mm × 22 mm length by width, thick and proximally rounded, with distal pointed tip and deep <3 mm wide notch on one side (Fig. 3g); large tooth at each end of notch, sometimes with 1–2 extra teeth at proximal end; typically single small tooth on outer margin; bract with smooth upper surface, typically concave and with bracteal canal scar originating on upper surface (no proximal diverticulum identifiable), passing over thickened proximal end of bract and onto ventral keel; latter with deep concavity on each side; bracteal canal thin, terminating in small apical button. Two immatures <15 mm × 13 mm length by width, with thick bracteal canal and scar, latter with short proximal diverticulum.

Gastrozooid, tentacle, and tentilla

Mature gastrozooid <9 mm × 2 mm length by width, column opaque and yellowish to translucent with hepatic stripes around mouth; basal basigaster pink, typically swollen (Fig. 3j); thick pinkish-grey annulate tentacle attached to basigaster on one side, with small white oval basal disc adjacent; tentacle much coiled, precluding measurement of length, and bearing large number of filamentous immature and mature tentilla (mostly obscured by siphosomal stem and basigaster in Fig. 3j); each mature tentillum arising from one annulus of tentacle, with cnidoband of 4–5 coils, sometimes quite loose, and single much coiled terminal filament with minute acorn-shaped cupulate process at tip (Fig. 3b); younger tentilla with more closely coiled cnidoband. Immature

gastrozooids 5 mm × 2 mm length by width, with basigaster longer relative to column (Fig. 3i).

Palpon and palpacle
Yellowish white < 15 mm × 2 mm length by width, typically with small swelling below tip, and collar of refringent nematocysts at tip (Fig. 3f); small proximal basal disc; palpacles of detached palpons mostly short and broken, longest measured 6 mm.

Gonophores and gonodendra
Regularly arranged on stem with 8–12+ male gonophores immediately distal of gastrozooid, then female gonodendron with 10–15+ female gonophores in each cormidium; palpons attached with gonophores. Male gonophores pink, with thin long pedicel when mature, largest approximately 3 mm long (Fig. 3l), four radial canals and four small tentacular buds (with nematocysts) at ostium. Female gonophores white, arranged in gonodendra, each connected to stem by thick pink gonodendron pedicel with opaque strengthening rings; approximately 15 gonophores per gonodendron, at varying stages of development and with nucleus discernible in some (Fig. 3k); gonophores ovoid, elongate with small ostium and four small tentacular buds; radial canals not discernible.

Discussion

The siphosome of the present 'Snellius' specimen of *Halistemma amphytridis* can be directly compared with those of the 'Albatross' specimens of *Stephanomia amphitridis* (Bigelow, 1911), as already noted above. These similarities, together with the newly described nectophores of *H. amphytridis*, are discussed and contrasted with the nectophores and siphosomal zooids, where known, of *H. cupulifera*, *H. rubrum*, *H. striata,* and *H. transliratum*.

Halistemma amphytridis nectophores are larger (20 × 22 mm length by width) than those of *H. rubrum* (13.0 mm × 10.5 mm length by width, Totton, 1965) and *H. transliratum* (13.0 mm × 13.4 mm length by width, Pugh & Youngbluth, 1988), and exhibit two vertical lateral ridges instead of one. In *H. rubrum* the single ridge passes diagonally from the apico-lateral ridge towards the infra-lateral ridge, but does not reach it (Totton, 1965, Fig. 21a), whereas in *H. transliratum* the single ridge joins the infra-lateral (Pugh & Youngbluth, 1988). In *H. amphytridis* the lateral ridge is incomplete, as it is in *H. rubrum*, but in the latter species the vertical lateral furrow, where the 'free end' of the lateral ridge originates, has a more diagonal alignment in lateral view (personal observation). In *H. transliratum* the lateral ridge is complete and arises from the apico-lateral ridge (Pugh & Youngbluth, 1988, Figs 1–3), whereas only a weak U-shaped and diagonally aligned vertical lateral furrow is discernible in the nectophores of the paratype (personal observation).

The nectophores of *Halistemma amphytridis* are only slightly larger than those of *H. striata* (16 mm × 19 mm length by width). In the latter species there is an extra complete vertical lateral ridge, and a weak vertical lateral furrow separating the most ostial vertical lateral ridge from the lateral ridge in the NHM material. A similar furrow is also figured in *H. striata* nectophores from the South Atlantic (Pugh, 1999, Fig. 3.12). In *H. striata* the lateral ridge divides into two branches; an upper branch passes to the lateral ostial chromatophore and a lower branch continues towards, but does not reach, the infra-lateral ridge. The course of this ridge is well shown by Totton (1965, Fig. 24b). Also in the NHM *H. striata* specimens, the two most ostially situated vertical lateral ridges reunite just before the junction with the infra-lateral ridge.

Two large *Halistemma* species noted in earlier literature and tentatively identified as *H. amphytridis* seem more likely referable to other species. Firstly, a 28–33 mm long nectophore illustrated by Daniel (1974, Fig. 3h) shows different proportions to the present nectophores, and the thrust block is narrow and divided, indicating that it might be an immature nectophore of another giant agalmatid. Secondly, Totton (1965) mentions material collected by Beebe and by the ships of the National Institute of Oceanography which may be *H. amphytridis*, quoted again in Mackie et al. (1987). However, in figures drawn from some of this material and supplied to the author by P. R. Pugh, the nectophore is much larger (< 33 mm × 33 mm length by width) than that of *H. amphytridis,* with

a vertical lateral ridge which divides into two branches, and the bract is differently shaped.

The course of the lateral radial canals over the nectosac in *Halistemma amphytridis* is more complex than in any other described species of the genus. In *H. transliratum* the dorsal loop is quite extensive (Pugh & Youngbluth, 1988), but neither it nor the ventral loop are as complex as the loops of *H. amphytridis* with their 'geometric' appearance and dorsal loop with an extra 'loop within a loop' (Figs 1b and c). In *H. rubrum* (Totton, 1954, Fig. 12) and *H. striata* (Pugh, 1999, Fig. 3.12) the course of the lateral radial canals is simpler. In *H. amphytridis* the nectosac widens ostially, and the upper part juts out over the lower region (Figs 1b and 2e) in a manner not apparent in other *Halistemma* species. The small ostio-lateral furrows in *H. amphytridis* (Fig. 2d) also occur in the *H. transliratum* paratype, and are similar to those observed in *Lychnagalma utricularia* (Pugh & Harbison, 1986, Fig. 2a).

Comparison of the two 'Albatross' specimens with the present siphosome confirms the identity of the present 'Snellius' specimen with *Stephanomia amphitridis* of Bigelow (1911). The dimensions and morphology of the 'Snellius' siphosomal elements, and their arrangement on the siphosomal stem fall within the range found in the 'Albatross' material, and fit the description and illustrations of Bigelow (1911). The bracts in Bigelow's pl. 18 Figures 5 and 8 correspond to the present dorsal bracts (Fig. 3h), the bract in pl. 18 Figure 7 corresponds to the present lateral bracts with a dorsal concavity (Fig. 3c and d), and the bract in pl. 18 Figure 6 has the same notch as the present ventral bracts, though it also has a proximal point. Furthermore, the eight ventral bracts identified in the two loaned USNM specimens have rounded proximal ends identical to those of the 'Snellius' ventral bracts (Fig. 3g). Lateral bracts in the loaned 'Albatross' material include both types found in the 'Snellius' specimen.

In the 'Albatross' material, the stem, basigasters of the gastrozooids and gonophores are varied shades of brown, whereas in the 'Snellius' specimen they are orange (stem, basigasters), pink (male gonophores), or white (female gonophores). This is probably due to preservation of the 'Albatross' specimens in alcohol, whereas in formalin pigment leaches out more readily (Mapstone, 2003), although a considerable amount still remains in the 'Snellius' specimen.

The arrangement of zooids within a single cormidium is more easily discerned in the 'Albatross' *Stephanomia amphitridis* specimens due to greater stem extension, but is identical to that in the more contracted 'Snellius' specimen. Bigelow clearly shows and describes in his text the sequence of zooids (excluding bracts) in one cormidium as follows: proximal gastrozooid (siphon) – male gonophores – female gonodendron (together with three-six associated palpons) – 3-6 mid-way palpons – 2-5 palpons proximal to next (more distal) gastrozooid – gastrozooid, or siphon (see Bigelow, 1911, pl. 18, Fig. 2 & p. 288). Bigelow (1911) also provides an excellent figure of a mature male gonophore (pl. 18, Fig. 4) which clearly shows the four tentacular rudiments on the bell margin. In the 'Snellius' specimen, there are four similar smaller rudiments around the ostium of the female gonophore (two are shown, but not labelled, in Fig. 3k). Although four radial canals are identified in the umbrella of the largest 'Snellius' male gonophores, they are omitted from Figure 3l.

The *Stephanomia amphitridis* specimen described by Huxley (1859) is similar to the 'Snellius' material, and Huxley's Figures of detached bracts can be equated with the 'Snellius' dorsal (pl. 6, Fig. 6), lateral (pl. 6 Figs 5, 5a and 6), and ventral (pl. 6, Fig. 6b) bracts. *S. amphitridis* is thus here also considered a synonym of *Halistemma amphytridis*, in agreement with Bigelow (1911) and Totton (1965). In *S. nereidum*, Haeckel's description of 3–5 teeth on the bracts and their 'less regular' arrangement on the stem correspond closely to the 'Snellius' specimen, as do the nectophores (see above). The 'free internodes' noted by Haeckel are probably due to greater relaxation of his specimen.

Huxley's *Stephanomia amphitridis* specimen was collected from off the east coast of Australia at 32° S, 155° 7′ E (J. Goodman, personal communication), a relatively temperate location. In contrast, both the 'Albatross' and 'Snellius' specimens come from tropical waters, as well as another specimen, recorded as *Cupulita amphitrites* by Bedot (1896), from the Bay of Ambon, off Ceram. Other specimens recorded as *H. amphytridis* not yet reliably identified as the present species, come from areas as far apart as Bermuda (Totton, 1936),

the Bahamas (Haddock & Case, 1999), and Sagami Bay, Japan (Hunt & Lindsay, 1999). It is therefore inappropriate to suggest a distributional range for *Halistemma amphytridis* at this time.

The siphosomal zooids of *Halistemma amphytridis* show several differences to those of other *Halistemma* species. In *H. rubrum* the bracts are smaller, tridentate, and have an elongate apical pit of nematocysts on the upper surface, as shown by Totton (1954, Fig. 13). The cormidial arrangement is also different, with male gonophores arising in three gonodendra at the bases of three palpons, distal to a single female gonodendron arising from the base of a single more proximal palpon (Totton, 1965). The tentillum of *H. rubrum* has a small cone-shaped coil at the tip of the terminal process, illustrated in detail by Pugh & Youngbluth (1988, Fig. 6d), and in relation to a whole gastrozooid tentacle by Vogt (1854, pl. 8, Fig. 6). In *H. transliratum* four bract types are identified by Pugh & Youngbluth (1988). One is the elongate immature nectalia-type not found in the mature 'Snellius' *H. amphytridis* specimen. The other three types are smaller, of which two have a transverse ridge unique to *H. transliratum*. The third bract type is superficially similar to the lateral bracts of *H. amphytridis*, but has an acutely pointed apex and more prominent lateral teeth than in *H. amphytridis*. Since only two specimens of *H. transliratum* have so far been described (Pugh & Youngbluth 1988), with male gonophores found only in the more mature holotype, it is possible that in this specimen the female gonophores had not yet formed. *H. transliratum* may eventually prove to be monoecious, like *H. rubrum* and *H. amphytridis*.

The tentillum of *Halistemma transliratum* bears a small swelling at the tip of the terminal filament. A larger acorn-like (cupulate) swelling or process is the only distinguishing character of the incompletely known species *H. cupulifera* (Lens & van Riemsdijk, 1908). In *H. transliratum*, the dark nematocyst-bearing basal portion of this process is smaller than the pale platelet-covered upper portion (Pugh & Youngbluth, 1988, Figs 6a and 7), whereas in *H. cupulifera* the basal portion is larger and the process is bigger (Pugh & Youngbluth, Fig. 6b). The slightly swollen tip of the terminal filament of *H. amphytridis* constitutes a similar, but smaller, bipartite cupulate process, with platelets distally and nematocysts basally (Fig. 3b). In *H. rubrum* and *H. transliratum* the cnidoband has 8–10 and 5–6 coils respectively (Totton, 1965; Pugh & Youngbluth, 1988), whereas in *H. amphytridis* it has only 4–5 (Fig. 3a). In the former two species these cnidobands appear to remain tightly coiled when mature, whereas in *H. amphytridis* the cnidoband becomes stretched at maturity to reveal two elastic ligaments (see also Bigelow, 1911, pl. 18, Fig. 3). These ligaments are also observed in *H. cupulifera* (Lens & van Riemsdijk, 1908).

Conclusions

The present 'Snellius' *Halistemma* specimen is referred to *H. amphytridis* because of the similarity of its siphosome with those of the 'Albatross' specimens. The nectophores are distinctive with two complete vertical lateral ridges and particularly complex lateral radial canals. The tentillum has a very small cupulate process at the tip of the terminal filament, smaller than found in other *Halistemma* species, and different also from the spiral tip to the terminal process of *H. rubrum*.

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Phylogeny and biogeography of *Anthopleura* in the North Atlantic Ocean

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Key words: *Bunodosoma*, sea anemone, morphology, Actiniaria

Abstract

Two species of *Anthopleura*, *A. ballii* and *A. thallia*, co-occur in Northern Europe with *Bunodosoma biscayensis*, a species closely allied to *Anthopleura*. The historical factors underlying their distribution are here investigated as part of a preliminary phylogenetic analysis of morphological data. Based on this analysis, *Bunodosoma* is nested within *Anthopleura*, and *B. biscayensis* is more closely related to members of *Anthopleura* than to other members of *Bunodosoma*. Because *B. biscayensis* also has adhesive verrucae, rather than non-adhesive, rounded vesicles, it is transferred to *Anthopleura*. The tree consists of two major clades, each of which includes species from the Atlantic and the Pacific. The tree shows geographic structure: one clade includes species found only in the Northern Hemisphere, and the other includes widespread species and species found only in the Southern Hemisphere. Although all of the European species of *Anthopleura* are part of the Northern Hemisphere clade, none are one another's closest relative.

Introduction

Members of the sea anemone genus *Anthopleura* are among the most familiar and well-known actiniarians. Species in this group have been used as a model system for understanding temperate rocky intertidal ecology and actiniarian biology (e.g. Bigger, 1980; Sebens, 1983; Francis, 1988; Harris, 1991; Kruger & Griffiths, 1998; Weis et al., 2002). One reason members of *Anthopleura* are often the focus of research is because members of this group tend to be locally abundant. In addition, *Anthopleura* tends to be locally diverse, with a single locality harboring multiple species belonging to the genus. This local diversity provides an ideal framework for comparative research, illuminating species-level differences in ecology, reproductive biology, or physiology.

This high local diversity is achieved in several ways. The fauna of the Galapagos Islands includes a widespread species, *Anthopleura nigrescens* (Verrill, 1869), and an endemic species, *Anthopleura mariscali* (Daly & Fautin, 2004). In Central California, the southern extent of the ranges of *Anthopleura artemisia* Pickering in (Dana, 1846), and *Anthopleura xanthogrammica* (Brandt, 1835), overlap with the northern extent of the ranges of *Anthopleura elegantissima* (Brandt, 1835), and *Anthopleura sola* (Pearse & Francis, 2000). The historical causes of local diversity are also varied; for example, both sympatric speciation and immigration have been implicated in Central California (McFadden et al., 1997; Pearse & Francis, 2000; Geller & Walton, 2001).

The *Anthopleura* fauna of the North Atlantic comprises two species, *Anthopleura ballii* (Cocks, 1851) and *Anthopleura thallia* (Gosse, 1854). Both of theses species are known only from the European coast; no species belonging to *Anthopleura* have been reported from the North Atlantic coast of North America. *Bunodosoma biscayensis* (Fischer, 1874), a species that has the anatomical attributes characteristic of *Anthopleura*, including acrorhagi and adhesive verrucae (den Hartog,

1987), is also found in the northeastern Atlantic. A phylogenetic analysis of morphological attributes is used to evaluate whether these species have a single or separate origin, and to explore the factors which might have had a role in shaping the diversity of *Anthopleura* in the North Atlantic.

Based on this analysis, the north Atlantic species are inferred to have separate origins. Because *A. ballii* is at the base of the tree, its phylogeographic affinities cannot be ascertained; the pattern of relationships among *Anthopleura* species supports either a trans-arctic or Atlantic origin for *A. thallia*. These data concur with earlier studies of molecular data (e.g. McCommas, 1991; Geller & Walton, 2001) in finding that *Anthopleura* is not monophyletic with respect to *Bunodosoma*. In all equally parsimonious trees, *B. biscayensis* nests with species of *Anthopleura* rather than with the other members of *Bunodosoma*; it is most closely related to species found in the Pacific rather than the sister taxon to either European species. In addition to its apparent phylogenetic relationship with species of *Anthopleura*, *B. biscayensis* has adhesive verrucae and acrorhagi, the two features that currently characterize *Anthopleura* (see England, 1987; Belém & Pinto, 1990), and lacks the non-adhesive vesicles characteristic of *Bunodosoma*.

Materials and methods

Taxa and characters

The taxon samples used by Geller & Walton (2001) and by McCommas (1991) were the starting point for this analysis to facilitate comparison of molecular and morphological results. McCommas considered a broad sample of taxa from the Gulf of Mexico and Caribbean, and Geller & Walton's (2001) hypothesis of relationship is the most recent and rigorous analysis of relationships among *Anthopleura*. The results of McCommas (1991) were used to define the ingroup taxa, and to choose an outgroup. All of the species sampled by Geller & Walton (2001) except *Anthopleura asiatica* Uchida & Muramatsu, 1958, were included in this analysis; the omission of *A. asiatica* was based on the availability of material for study. In addition, I included the European species *A. ballii*, *A. thallia*, and *B. biscayensis*, the Chilean species *Anthopleura hermaphroditica* (Carlgren, 1899), and two new species, *Anthopleura mariscali* (Daly & Fautin, 2004), from the Galápagos Archipelago and *Anthopleura a-us* Yanagi & Daly (in press) from the east coast of Japan.

For these 21 ingroup taxa, I examined 32 attributes, including aspects of external anatomy, muscle histology, cnidom, and biology (Appendix 1). Observations and photographs of live specimens were used wherever possible. Internal anatomy and histology were explored using dissections and serial sections, where appropriate. All species not known to brood and for which none of the examined specimens contained juveniles were assumed to broadcast spawn, and all species for which the examined specimens contained gametic tissue of a single sex were assumed to be gonochoric.

Analysis

The morphological character matrix was compiled in Winclada (Nixon, 2002), and analyzed in NONA (Goloboff, 1999). The search strategy employed a branch swapping with tree-bisection and reconnection algorithm for 1000 replications, holding two trees at each step with a maximum of 100 000 trees held (NONA commands mult * 1000, max*, hold 100,000/2). All characters were unordered and weighted equally. Decay values (Bremer, 1988) for each node were assessed using NONA (Goloboff, 1999). *Epiactis prolifera* (Verrill, 1869), was used to root the trees; this species belongs to the Actiniidae but is clearly not part of the complex of species that belong to *Anthopleura* and *Bunodosoma* (see McCommas, 1991).

Results

Twelve trees of 100 steps, with a CI = 0. 36 and RI = 0.56, were recovered. The tree consists of two large clades, with *A. ballii* at the base (Fig. 1). The larger of these includes all species restricted to the Northern hemisphere, and is heretofore called the Northern clade. The other, smaller clade contains the widespread species *A. nigrescens* and *Anthopleura krebsi* (Duchassaing & Michelotti, 1860), the Southern Pacific species *Anthopleura handi* (Dunn, 1978), and the South American species *A. mariscali* and *A. hermaphroditica*.

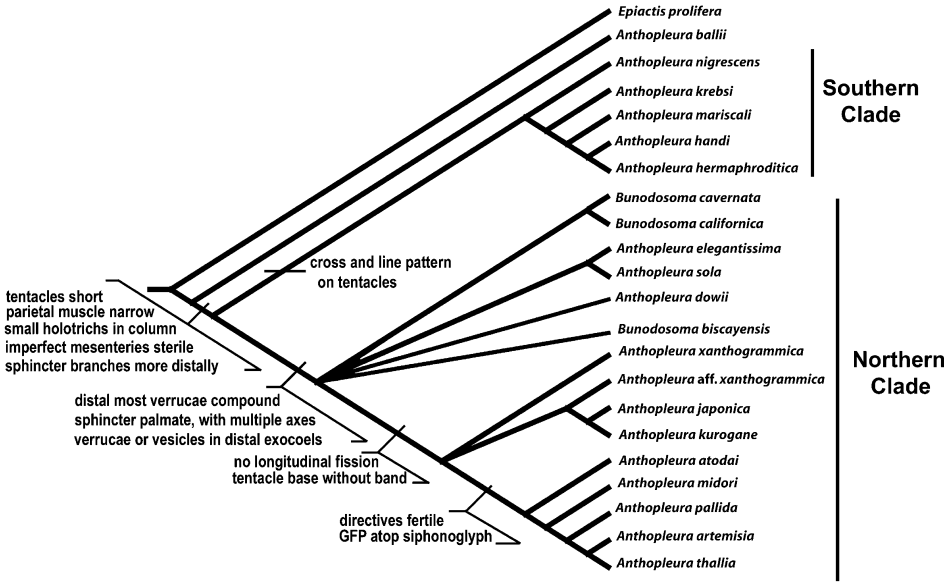

Figure 1. Strict consensus of 12 equally parsimonious trees, L = 100; CI = 0.36; RI = 0.56 Values above the nodes are decay indices.

The twelve primary trees differ with respect to the position of *Anthopleura dowii* (Verrill, 1869), and the clade that includes *Bunodosoma cavernata* (Bosc, 1802) and *Bunodosoma californica* (Carlgren, 1951). In some of the primary trees, *A. dowii*, *B. cavernata* + *B. californica*, and a clade consisting of *A. elegantissima* and *A. sola* group together; in other trees either *A. dowii* or the *B. cavernata* + *B. californica* clade group with *A. elegantissima* + *A. sola*. In one of the twelve trees, the *B. cavernata* + *B. californica* clade is at the base of the Northern clade.

Anthopleura ballii is the basal-most ingroup taxon, the sister group to the two clades containing all of the other species. In all trees except the one mentioned above, *A. biscayensis* is at the base of the Northern clade. *Anthopleura thallia* nests within the Northern clade, as the sister group to *A. artemisia*, a Pacific species.

Discussion

Biogeography of Anthopleura

According to this analysis, the primary geographic structure of *Anthopleura* is North–South, rather than Atlantic–Pacific. The Southern Hemisphere species are inferred to be the descendants of widespread taxa. The distributions of *A. krebsi* and *A. nigrescens* span the equator in the Atlantic and Pacific respectively (see Duchassaing & Michelotti, 1860; Verrill, 1869; Carlgren & Hedgpeth, 1952; Dunn, 1974; England, 1987; Belém & Pinto, 1990), implying a tropical origin for the clade that includes widespread and Southern hemisphere species. Because *A. ballii* is a Northern hemisphere species, and is the sister group to a clade which is subdivided into a clade of Northern species and one with an implied pan-tropical origin, the Northern hemisphere seems to be the center of origin for the group.

The North Atlantic species of *Anthopleura* are not one another's closest relatives, and therefore not the result of a single radiation. Two of the European species, *A. ballii* and *A. biscayensis*, are reconstructed at the base of the clade which contains them, making determinations of their origin difficult. However, in the case of *A. biscayensis*, a Pacific origin seems likely, as it is always the sister group to a clade or species from the Pacific.

The biogeographic origin of *A. thallia* is also difficult to interpret. The sister taxon of *A. thallia* is *A. artemisia*, a Pacific species, but the sister taxon of the *A. artemisia* + *A. thallia* clade is *Anthopleura pallida* Duchassaing & Michelotti, 1864, a species found in the Gulf of Mexico. This series of relationships implies two equally

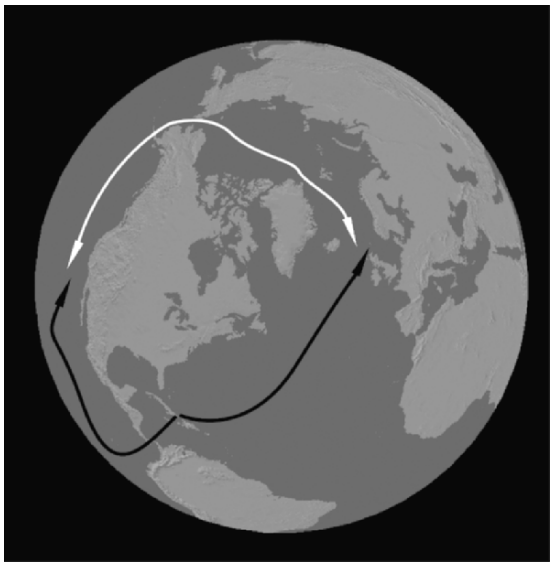

Figure 2. Possible dispersal routes for *Anthopleura artemisia* and *Anthopleura thallia*. Both species may have dispersed from a common ancestor that lived in the Caribbean (black lines). Alternatively, the ancestor to *A. artemisia* and *A. thallia* may have dispersed from the Caribbean into the Pacific (or Atlantic) and then across the Arctic (white line) into the Atlantic (or Pacific).

parsimonious scenarios (Fig. 2). *Anthopleura thallia* and *A. artemisia* may have each dispersed from an ancestor in the Gulf of Mexico. Alternatively, the ancestor of *A. artemisia* and *A. thallia* may have dispersed into either the Atlantic (or Pacific), with a subsequent dispersal into the Pacific (or Atlantic).

Although these conclusions are at odds with those of Geller & Walton (2001), the differences are likely the result of differences in taxonomic sampling rather than fundamental differences in the phylogenetic signal of morphological and molecular data. The primary difference is that in their tree, *A. handi* and *A. nigrescens* are the sister group to the clade containing *A. artemisia* and all of the Japanese species, rather than outside a clade containing *A. artemisia*, and *A. elegantissima*, *A. sola*, *A. xanthogrammica*, and all the Japanese species of *Antholpleura*. However, several nodes are congruent, including the grouping of *A. nigrescens* and *A. handi*, and the placement of *A. artemisia* as the sister taxon of a clade consisting of *A. japonica* (Verrill, 1899), *A. kurogane* (Uchida & Muramatsu, 1958), and *Anthopleura* aff. *xanthogrammica* (called *Anthopleura* sp. by Geller & Walton, 2001). The east-west split Geller & Walton (2001) noted for the Northern Pacific species they studied is also seen in the morphological analysis if the Atlantic species are pruned out: *A. artemisia* groups with the Japanese species, with *A. elegantiisima*, *A. sola*, and *A. xanthogrammica* outside.

These results are preliminary in that the taxon sample does not include all described species of *Anthopleura*. In particular, additional Southern Hemisphere species need to be included to test the robustness of the North-South pattern.

Evolution and taxonomy of Anthopleura

This tree provides insight into morphological evolution within *Anthopleura*, and highlights characters and taxa which deserve further investigation. Several features commonly used to diagnose and differentiate species within *Anthopleura* are homoplastic. Oral disc pattern, for example, may be useful for distinguishing species, but the pattern of nested rings of alternating light and dark splotches seen in *A. artemisia* and *A. hermaphroditica* are inferred to have arisen independently. The number of mesentery cycles shows no discernible phylogenetic pattern, but may indicate something about ecology and biogeography: all of the species with five cycles of mesenteries are found on the Pacific coast of North America. Based on this analysis, the retractor muscle extends onto the lamellae between the retractor and parietal muscles several times within this group. In contrast, the shapes of the marginal sphincter and parietal muscle are phylogenetically informative, as is the fertility pattern of mesenteries. The morphology of the sphincter shows an intriguing evolutionary pattern: the transition from being more developed on the side closer to the oral disc to being more developed on the opposite side passes through the intermediate state of being equally developed on both sides.

Homoplasy in these characters may indicate that these features are under ecological, functional, or some other constraint. Alternatively, the problem may be with the delimitation of the character, rather than the feature itself. For example, the oral disc pattern of *A. hermaphroditica* and *A. artemisia* may be achieved through the combination of different elements that appear superficially similar in

the aggregate but which differ in their particulars. This initial assessment provides guideposts for refining and revising the description and delimitation of morphological features.

Biological features like reproductive mode and sexuality are largely uninformative, characterizing either small terminal clades or single species. However, the inferred patterns of evolution for both longitudinal fission and symbiotic state are more complex, and merit further attention. The ability to undergo longitudinal fission is inferred as ancestral for the ingroup, and is reconstructed as lost in three instances: in *A. hermaphroditica*, in *A. sola*, and in the clade that includes *A. xanthogrammica* as its most basal member. Fission reappears twice within the clade characterized as having lost it: in *A. japonica* and *A. thallia*. The opposite pattern is seen with respect to symbiosis: the acquisition of symbionts is a novelty in three instances (*A. hermaphroditica*, *B. cavernata*, and the clade that includes *A. dowii*). Symbionts are secondarily lost in the clade that includes *A. midori* as its most basal member.

The traits by which *Anthopleura* are currently recognized, acrorhagi and adhesive verrucae, are shared primitive features (sympliesiomorphies). Based on this tree, verrucae are inferred to be transformed into vesicles in the clade containing *B. cavernata* and *B. californica*. These non-adhesive, rounded vesicles are a shared derived characteristic (synapomorphy) for *Bunodosoma*; *A. biscayensis* lacks this synapomorphy, having instead the primitive condition of adhesive verrucae. The implied directionality of the transformation between verrucae and vesicles is intriguing, as it suggests a transformation from a structure with functional significance (i.e. retarding water loss: Hart & Crowe, 1977) to one without a clear function.

From a taxonomic standpoint, transferring *B. biscayensis* to *Anthopleura* ensures that the features seen in the species are compatible with those considered diagnostic of its genus (see England, 1987; Belém & Pinto, 1990). Transferring *B. biscayensis* from *Bunodosoma* to *Anthopleura* makes practical sense, but because the concept of *Anthopleura* is so poorly defined, placement in *Anthopleura* should be recognized as carrying little phylogenetic significance. For example, *A. ballii* may not belong in this group; its placement at the base of the tree is indicative of its separation from the other species in *Anthopleura* and *Bunodosoma*. Unlike the other species of *Anthopleura* (and of *Bunodosoma*), *A. ballii* has long, gracile tentacles that are not fully retractile, a weakly muscled column, and fertile imperfect mesenteries; it also lacks small holotrichs (called hetrotrichs by England, 1987) in the column. However, as there is no other genus to which this species can readily be assigned, it remains in *Anthopleura*.

Anthopleura is polyphyletic; in all primary trees the clade containing *B. cavernata* and *B. californica* is within the clade containing the named species of *Anthopleura*. In order to make *Anthopleura* monophyletic, *Bunododosma* would have to be submerged within it, or *Anthopleura* would have to be divided into several genera. However, pending more detailed studies of all nominal species of *Anthopleura*, *Bunodosoma* should continue to be recognized as a separate genus, because the *Bunodosoma* clade cannot be placed unambiguously and has a clear synapomorphy.

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Appendix 1. Character list and matrix of morphological features analyzed in this study

	1–5	6–10	11–15	16–20	21–25	26–30	31–32
E. prolifera	00101	100??	-?010	001-0	01101	000?0	10
B. biscayensis	00000	01111	01010	11001	10010	10101	11
B. cavernata	00021	01011	1?011	11--	100--	-1101	10
B. californica	00121	01011	1?020	11101	10010	10001	10
A. artemisia	11010	01100	00120	11000	00011	10101	11
A. a-us	00001	0111-	01111	11100	01111	11111	11
A. balli	10121	01100	01010	00000	1001-	01001	01
A. dowii	00011	01111	01020	11001	10010	11111	11
A. elegantissima	00001	01110	01020	10101	10010	11111	11
A. handi	01011	01100	00000	??001	11010	00111	11
A. hermaphroditica	01011	01100	00011	01101	01110	11111	11
A. japonica	01021	01111	00010	0000-	10011	11111	11
A. krebsi	00011	01100	01011	12011	10010	10111	11
A. kurogane	00120	01111	00010	02101	00011	11111	11
A. mariscali	10011	01100	00011	00001	10010	10111	11
A. midori	10010	01101	01110	12000	00011	10111	11
A. nigrescens	0001?	01100	01010	01101	1001?	10101	11
A. pallida	11011	01100	00110	??001	00011	10111	11
A. sola	00001	01110	01020	10101	00010	11111	11
A. thallia	11010	01100	00112	??100	10011	00101	11
A. aff. xanthogrammica	00000	01111	01010	00000	00010	11111	11
A. xanthogrammica	00000	01111	01020	11000	00010	11111	11

Character list

1. Column shape: stout = 0; elongate = 1.
2. Oral disc pattern: rays = 0; nested, alternating squares around mouth = 1.
3. Tentacle length: approximately equal to oral disc radius, less than oral disc diameter = 0; longer than oral disc diameter = 1.
4. Pattern on tentacles: none = 0; longitudinal line with cross bars = 1; random flecks = 2.
5. Base of tentacle: unmarked = 0; opaque band = 1.
6. Marginal projections: present = 0; absent = 1.
7. Acrorhagi: absent = 0; present = 1.
8. Endcoelic columnar protrusions: absent = 0; present = 1.
9. Hollow, exocoelic columnar protrusions: absent = 0; present distally = 1.
10. Shape of distal-most columnar protrusions: simple = 0; compound = 1.
11. Histology of columnar protrusions: apex without cnidae or granular gland cells (verrucae) = 0; apex with cnidae and granular gland cells (vesicles) = 1.
12. Arrangement of columnar protrusions: extend 2/3 of way from from distal margin to limbus = 0; extend from distal margin to limbus = 1.
13. Siphonoglyph marked with green florescent protein: no = 0; yes = 1.
14. Number of cycles of mesenteries: 3 = 0; 4 = 1; 5 = 2.
15. Shape of marginal sphincter muscle: palmate = 0; pinnate = 1; diffuse = 2.
16. Number of lamellae in marginal sphincter muscle: 1 = 0; 2 = 1.
17. Symmetry of marginal sphincter muscle: symmetrical = 0; asymmetrical, more developed distally = 1; asymmetrical, more developed proximally = 2.
18. Lamella of mesentery muscular between retractor and parietal: no = 0; yes = 1.
19. Retractor muscle with accessory muscles: no = 0; yes = 1.
20. Parietal muscle shape: broad = 0; narrow = 1.
21. Longitudinal fission: no = 0; yes = 1.
22. Reproductive mode: broadcast spawning = 0; brooding = 1.
23. Sexuality: gonochoric = 0; hermaphroditic = 1.
24. Non-directive perfect mesenteries: sterile = 0; fertile = 1.
25. Directive mesenteries: sterile = 0; fertile = 1.

26. Imperfect mesenteries: fertile = 0; sterile = 1.
27. Zooxanthellae: absent = 0; present = 1.
28. Small holotrichs (heterotrichs *sensu* England, 1987) in column: absent = 0; present = 1.
29. Holotrichs in acrorhagi dimorphic: no = 0; yes = 1.
30. Large microbasic *b*-mastigophores in filament: absent = 0; present = 1.
31. Small microbasic *b*-mastigophores in filament: no = 0; yes = 1.
32. Columnar protrusions adhesive: no = 0; yes = 1.

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Some implications of molecular phylogenetics for understanding biodiversity in jellyfishes, with emphasis on Scyphozoa

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Key words: conservation, Cubozoa, evolution and development, Hydrozoa, morphology, cryptic species

Abstract

Statistical phylogenetic analyses of 111 5.8S and partial-28S ribosomal DNA sequences (total aligned length = 434 nucleotides) including jellyfishes representing approximately 14% of known scyphozoan morphospecies (21% genera, 62% families, and 100% orders) are presented. These analyses indicate stauromedusae constitute a fifth cnidarian class (Staurozoa) basal to a monophyletic Medusozoa (= Cubozoa, Hydrozoa, and Scyphozoa). Phylogenetic relationships among the medusozoans are generally poorly resolved, but support is found for reciprocal monophyly of the Cubozoa, Hydrozoa, Coronatae, and Discomedusae (= Semaeostomeae + Rhizostomeae). In addition, a survey of pairwise sequence differences in Internal Transcribed Spacer One within morphospecies indicates that scyphozoan species diversity may be approximately twice recent estimates based on morphological analyses. These results highlight difficulties with traditional morphological treatments including terminology that obfuscates homologies. By integrating molecular phylogenetic analyses with old and new morphological, behavioural, developmental, physiological, and other data, a much richer understanding of the biodiversity and evolution of jellyfishes is achievable.

Introduction

Approximately 200 species of Scyphozoa are currently recognized (Mianzan & Cornelius, 1999), a number that has fluctuated historically and depends on the publication consulted (e.g. Kramp, 1961; Franc, 1995; Mianzan & Cornelius, 1999). The estimate is also influenced, to a small extent, by the still debated status of box-jellyfish as an order (Cubomedusae) within the Scyphozoa (e.g. Haeckel, 1879) or as an autonomous class (the Cubozoa; Werner, 1975). Irrespective of the precise number, scyphozoan species diversity appears low relative to the number of species described in other cnidarian classes: approximately 9000 species of Anthozoa (Brusca & Brusca, 2003) and perhaps over 3000 species of Hydrozoa (Schuchert, 1998). The low diversity purportedly reflects low levels of endemism and a high proportion of cosmopolitan species (Mianzan & Cornelius, 1999).

Recent molecular analyses (e.g. Bridge et al., 1995; Dawson & Martin, 2001; Collins, 2002), however, suggest sometimes different phylogenetic relationships, species boundaries, and diversity of jellyfishes, indicating difficulties in interpreting the taxonomic significance of relatively few traditional morphological features that are rarely diagnostic (e.g. Dawson, 2003). This problem is long-standing, as indicated by Mayer (1910, p. 596):

> world-wide forms of medusae, growth-stages, colour varieties and local races have frequently been described as separate species, but as our

> knowledge increases many intergrading forms come to light thus reducing the so-called species to a few dominant types with numerous, closely related offshoots. It is unfortunate that the aim of the old systematic zoology was mainly toward the emphasising of distinctions rather than the indication of affinities and the discovery of relationships.

Mayer's (1910) phylogenetic perspective emphasised the inter-connectedness of organisms through modification by descent, but concomitantly diminished the perceived taxonomic import of variation, blurring previously proposed species boundaries. Similarity in an evolutionary context subsequently translated easily into synonymy in more taxonomically oriented treatments (e.g. Kramp, 1961). In itself, Mayer's advocacy of phylogeny was progressive, but dealing with organisms that have few useful morphological characters in the absence of objective criteria, quantitative data, and late-20th century statistical tools, some valid species were inevitably overlooked.

In this context, molecular data make two major contributions. They provide many additional characters, thereby increasing the chance that groups will be differentiated, and they allow phylogenetic reconstruction independent of morphological characters, thereby promoting analyses of morphological evolution. Here, I present a molecular phylogenetic framework, using nuclear ribosomal DNA, for interpreting evolutionary patterns and biodiversity in the jellyfishes, and discuss some of its implications at class, order, and species levels.

Materials and methods

Tissue from the bell margin, gonad, gut, or oral arms of medusae (Table 1) was placed in excess DMSO + NaCl solution or >70% ethanol (Dawson et al., 1998), and stored in a refrigerator or freezer before and after shipping. Total DNA was extracted using a CTAB extraction protocol (Dawson et al., 1998; Dawson & Jacobs, 2001). PCR using *Taq* Polymerase (Perkin Elmer), and MJ Research MiniCyclers or Perkin Elmer GeneAmp 9700 s, began with an 8 min denaturation at 94 °C, 2 min annealing at 48 °C, 3 min extension at 72 °C, 4 min denaturation at 94 °C, 2 min annealing at 49 °C, 2 min extension at 72 °C, followed by 33 cycles, each consisting of 45 s at 94 °C, 45 s at 50 °C, and 90 s at 72 °C; PCRs terminated with a 10 min extension step (72 °C), then refrigeration (4 °C). Nuclear ribosomal DNA including the entire Internal Transcribed Spacer One (ITS1), 5.8S, ITS2, and the 5′ portion of 28S, was amplified using the primers jfITS-5f (5′-ggtttccgtaggtgaacctgcggaaggatc-3′) and 28S-2R (5′-gctttgggctgcagtcccaagcaacccactc-3′). Amplified fragments were cloned (Invitrogen TOPO TA cloning kit), purified (Qiagen DNEasy or Pharmacia Flexiprep kits), fluorescently labelled with BigDye using the PCR or Invitrogen's M13Reverse and M13Forward primers, and sequenced on Applied Biosystems 373 Autosequencers.

Sequences were confirmed as nuclear rDNA and ITS regions by BLAST comparison with sequences in GenBank (Altschul et al., 1997). ITS regions were excluded due to difficulties with sequence alignment, leaving 5.8S and 28S regions to be aligned by eye using Se-Al v1.d1 (Rambaut, 1995) on a Macintosh PowerBook G3.

Phylogenetic analyses employed maximum parsimony (MP), maximum likelihood (ML), and distance approaches in PAUP* 4.0b10 (Swofford, 2002). MP employed two weighting schemes: (1) 'unweighted,' and (2) the reciprocal of occurrence frequency. Gaps were coded as a fifth character. The heuristic search used 10 000 replicates and tree-bisection-reconnection (TBR). ML analyses via Quartet Puzzling (10 000 steps: Strimmer & von Haeseler, 1996) used the general time reversible (GTR) model with among-site rate variation (proportion of invariable sites = 0.3, Gamma distribution shape parameter = 0.5) and equal base frequencies, equivalent to the TrNef + I + G model (MODEL TEST: Posada & Crandall, 1998). Distance analyses used GTR + I + G (proportion of invariable sites = 0.3, Gamma distribution shape parameter = 0.5) with TBR and the minimum evolution (ME) optimality criterion. Bootstrap analyses used the same search options as described above for MP (1000 bootstrap replicates, except 10 heuristic searches per replicate) and ME (100 replicates).

ITS1 sequences of morphospecies sampled from ≥2 locations and of *Nausithoe punctata*

Table 1. Taxonomy and sample locations of specimens

Class	Order	Family	Genus	Species	Location
Hydrozoa	Cystonectae	Physaliidae	*Physalia*	*physalis* Linné	Hawaii (Kauai), Australia (Bribie Island)
	Leptomedusae	Aequoreidae	*Aequorea*	*aequorea* (Forskål)	Alaska (Kachemak Bay), California (Morro Bay), South Africa (Hout Bay[a])
			Aequorea	–	Australia (Darling Harbour, Noosa Heads, Port Douglas), Palau
		Eirenidae	*Eutonina*	*indicans* (Romanes)	Alaska (Kachemak Bay)
		Mitrocomidae	*Mitrocomella*	*grandis* Kramp	South Africa (Hout Bay)
	Limnomedusae	Proboscidactylidae	*Proboscidactyla*	*menoni* Pages, Bouillon & Gili	South Africa (Hout Bay)
	Milleporina	Milleporidae	*Millepora*	*exaesa*	GenBank (U65484)
	Narcomedusae	Aeginidae	*Solmundella*	*bitentaculata* (Quoy & Gaimard)	Palau (Risong)
	Stylasterina	Stylasteridae	*Distichopora*	sp.	GenBank (U65483)
Cubozoa	Cubomedusae	Chirodropidae	*Chironex*	*fleckeri* Southcott	GenBank (U65477)
			Chiropsalmus	*quadrigatus* Haeckel	Palau (Risong)
Scyphozoa	Coronatae	Atollidae	*Atolla*	*vanhoeffeni* Russell	Southern California Bight
		Linuchidae	*Linuche*	*aquila* Haeckel	Palau, PNG[b] (Julian Reef)
		Nausithoidae	*Nausithoe*	*punctata* Kölliker	Palau (Risong)
			Nausithoe	*racemosa* (Komai)	Palau (Risong, Ulong), PNG (Duchess Island, Observation Point)
		Periphyllidae	*Periphylla*	*periphylla* (Péron & Lesueur)	Norway (Sognefjorden), Malvinas[c]
	Rhizostomeae	Cassiopeidae	*Cassiopea*	*andromeda* (Forskål)	PNG[d] (Emona, Observation Point)
			Cassiopea	*xamachana* R. P. Bigelow	USA (Florida)[d], Bermuda[d]
		Catostylidae	*Catostylus*	*mosaicus* L. Agassiz	Australia (Mooloolaba)
		Mastigiidae	*Mastigias*	*papua* (Lesson)	Palau (Ongael Lake, Clear Lake), Berau (Kakaban), PNG (Tufi)
			CRCN1524	–	Palau (Ulong)[e]
			Thysanostoma	*thysanura* Haeckel	Palau (Short Drop Off)
		Unidentified	–	–	Australia (Port Douglas)
	Semaeostomeae	Cyaneidae	*Cyanea*	*capillata* (Linné)	Alaska (Kachemak Bay), Australia (Merimbula Lake, Huon Estuary), GenBank (U65483), Norway (Raunefjorden)

Continued on p. 252

Table 1. (Continued)

Class	Order	Family	Genus	Species	Location
		Pelagiidae	*Chrysaora*	sp.	Australia (Noosa Heads)
		Ulmaridae	*Aurelia*	*aurita* (Linné)	Sweden (Bjornsund), USA (Rhode Island)
			Aurelia	*labiata* Chamisso & Eysenhardt	Alaska (Kachemak Bay), Canada (Sooke Inlet)
			Aurelia	*limbata* (Brandt)	Alaska (Kachemak Bay), Japan (Hokkaido)
			Aurelia	sp. 1/UBI	Australia (Perth, Queensland, Sydney), Japan (Miyazu Bay, Sakata Bay), USA (Long Beach, Marina del Rey)
			Aurelia	sp. 2	Brazil (São Paulo)
			Aurelia	sp. 3	Palau (Malakal)
			Aurelia	sp. 4	Berau (Kakaban), Hawaii (Oahu), Palau (Ongael, Hotwater)
			Aurelia	sp. 5/MKL	Croatia (Mljet Lake)
			Aurelia	sp. 6	Palau (Helen Reef)
			Aurelia	sp.	Adriatic Sea (North Adriatic, Bay of Ston)
			Phacellophora	*camtschatica* Brandt	USA (Morro Bay)
			Tiburonia	*granrojo* Matsumoto, Raskoff & Lindsay	GenBank (AY149900)
	Stauromedusae	Depastridae	*Depastromorpha*	*africana* Carlgren	South Africa (False Bay)
		Lucernariidae	*Haliclystus*	*auricula* (Rathke)	Chile (Los Molinos[f])

Specimens were identified to species using morphological and distributional information (except *Aurelia* spp.1–6; see Dawson, 2003). If species-level identification was not possible, specimens were identified to genus. Species identifications and taxonomy were based on Uchida (1947), Massin & Tomascik (1996), Arai (1997), Wrobel & Mills (1998), Bouillon (1999), Mianzan & Cornelius (1999), and Pugh (1999). Suborders within Rhizostomeae and subfamilies within Ulmaridae (Mianzan & Cornelius, 1999) are ignored because equivalent taxonomic ranks are not commonly recognized within other orders of Scyphozoa. A taxonomy based on Class Hydrozoa (e.g. Mianzan & Cornelius, 1999; Pugh, 1999) is used rather than the alternative scheme based on Superclass Hydrozoa (Bouillon, 1999). For context, there are currently estimated to be approximately 200 species, 69 genera, and 19 families in four orders of Scyphozoa, and approximately 20 species, eight genera, two families, in one order of Cubozoa. Thus this study covers approximately 14% of species, 21% of genera, 62% of families, and 100% of orders that have, at one time or another, been classified in Scyphozoa.

[a] Atlantic Ocean near Cape Town.
[b] Papua New Guinea.
[c] Falkland Islands.
[d] Following identifications of Holland et al. (in press).
[e] Coral Reef Research Foundation, invertebrate reference collection number.
[f] Near Valdivia.

(which included two morphotypes – var. 1 and var. 2 – from one location, L. Martin, pers. commun.) were aligned using ClustalX. Pairwise sequence differences within-morphospecies and within-and-between-regions were calculated in PAUP* 4.0b10.

Results

A total of 111 sequences, 102 new and nine from GenBank, with a total aligned length of 434 nucleotides (159 5.8S and 275 28S), including representatives from all currently recognized classes of Cnidaria and orders of Scyphozoa were analysed. 5.8S was unavailable for three sequences (*C. quadrigatus*, *M. papua* [Tufi], *T. granrojo*) and 5.8S or 28S was partial in another 17 (with generally ≤50 nucleotides missing). Analyses excluding and including the incomplete sequences recovered the same groups so the latter are presented here. There was no significant bias in nucleotide frequency (C 22.1%, T 24.4%, A 27.3%, G 26.2%; $p > 0.05$, MODEL TEST) and transitions were twice as frequent as transversions.

Hydrozoa, cubomedusae, Coronatae, Rhizostomeae, stauromedusae, Leptomedusae, and Mastigiidae were monophyletic in all analyses. Semaeostomeae was either reciprocally monophyletic (MP) or paraphyletic (ME, ML) with respect to Rhizostomeae. Scyphozoa, regardless of the taxonomic status of cubomedusae (i.e. as Cubomedusae or Cubozoa), was paraphyletic with respect to Hydrozoa in MP and ML analyses (Fig. 1). *Nausithoe* may be polyphyletic. Pairwise sequence differences within-species-between-regions ranged from 0 to 39%, while differences within regions varied from 0 to 17% (Table 2).

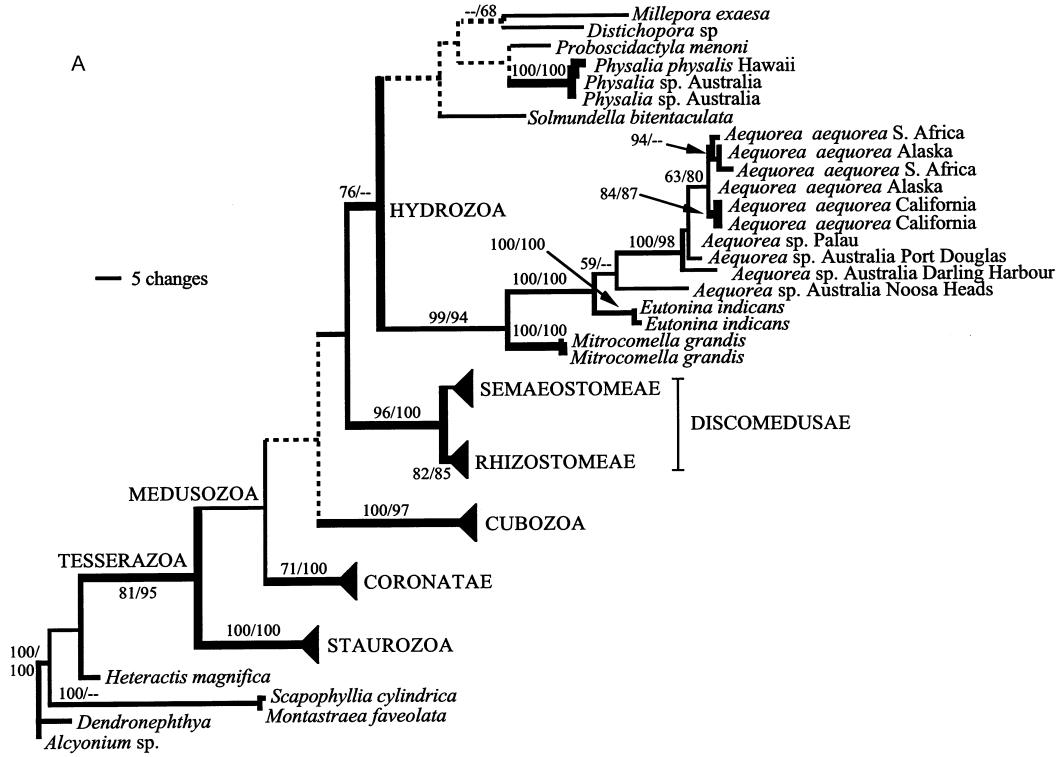

Figure 1. Ribosomal DNA (5.8S plus partial 28S) gene tree of medusozoans rooted with anthozoans. (A) Overview of relationships among classes and (B) details of relationships among species historically classified, at one time or another, as scyphomedusae. Tree shape shows one of 1407 most parsimonious trees (from 10^4 heuristic searches, ti:tv = 0.5 or 1), length = 684, CI = 0.475. Dashed branches were absent from the strict consensus. Thickened branches were also present in the maximum likelihood quartet puzzling tree, and the thickest branches were additionally present in the minimum evolution (ME) tree. Bootstrap values are indicated for branches with >50% bootstrap support using maximum parsimony or ME criteria, respectively (–, support <51% for that analysis).

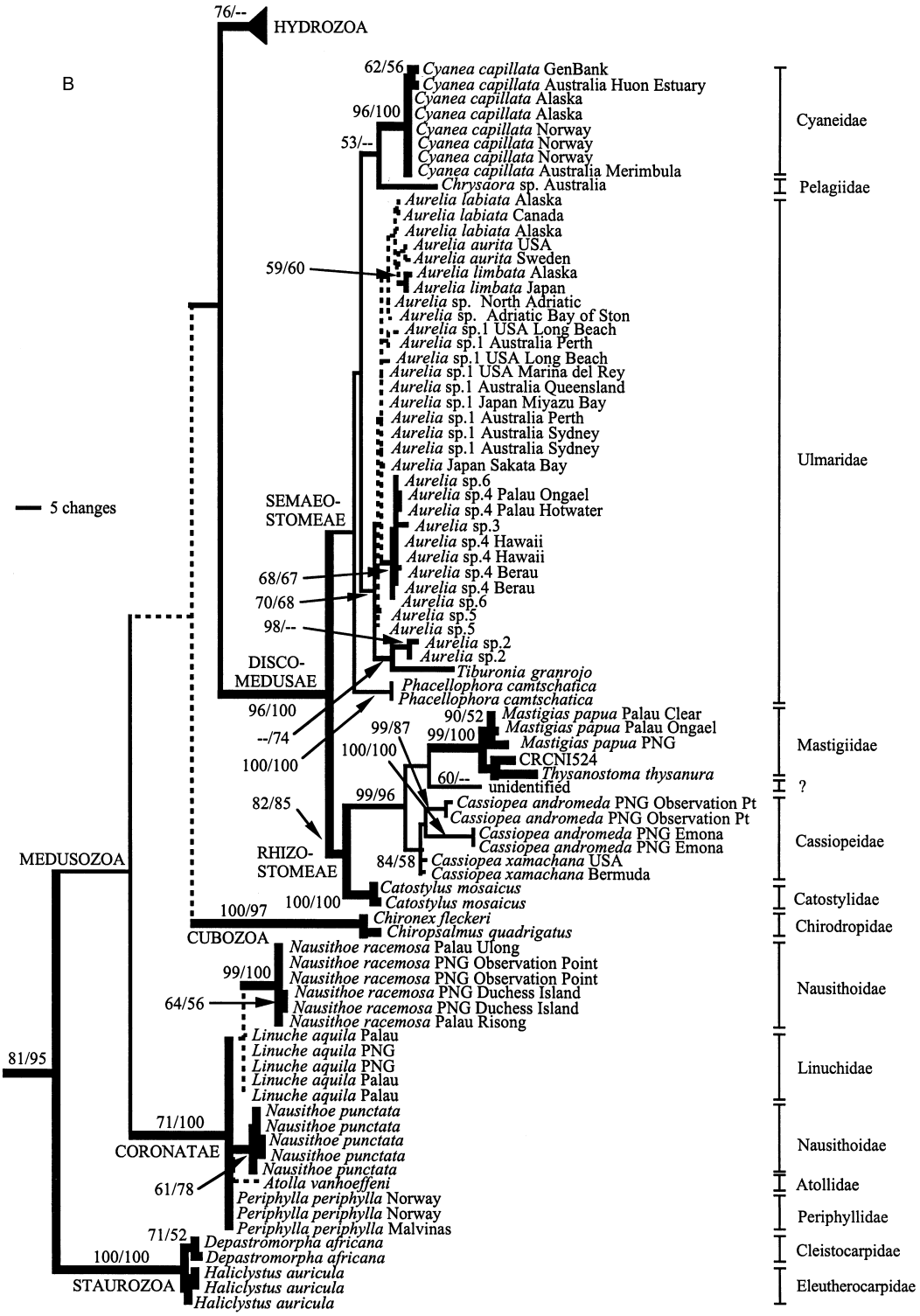

Figure 1. (Continued)

Discussion

Diversity at species-level and above

Phylogenetic analyses of 18S (Collins, 2002), 5.8S, and partial 28S (Fig. 1) support monophyly of classes Cubozoa and Hydrozoa, and scyphozoan orders Coronatae, Rhizostomeae, and Stauromedusae, as recognized by traditional morphological taxonomy. However, current taxonomic nomenclature does not appear to portray accurately some other phylogenetic relationships in the Cnidaria. Scyphozoa is paraphyletic with respect to both Cubozoa and Hydrozoa, due to the well-supported basal position of stauromedusae, and Semaeostomeae may be paraphyletic with respect to Rhizostomeae (see also Collins, 2002).

Thus, it would be useful to establish mutually consistent terms that facilitate evolutionary discourse. In this context, Tesserazoa (Salvini-Plawen, 1978) may be most appropriate for all non-anthozoan cnidarians (Collins, 2002),

Table 2. Percent pairwise sequence difference (PSD%) in Internal Transcribed Spacer One (ITS1) within morphospecies, between and within regions

Species	Regions	PSD %		Region	PSD %
		Mean	S.D.		
Aequorea aequorea					
	Alaska versus California	7.52	1.01	Alaska	0.00
	Alaska versus South Africa	3.73	0.12	South Africa	0.00
	California versus South Africa	7.34	0.34	California	3.41
Cassiopea andromeda					
	Emona versus Observation Point	39.19	0.88	Emona	0.30
	–	–	–	Observation Pt.	1.83
Cyanea capillata					
	Alaska versus Norway	0.91	0.18	Alaska	0.00
	Alaska versus Australia	12.14	0.80	Norway[a]	0.42
	Norway versus Australia	12.46	1.27	Australia	16.91
Mastigias papua					
	Palau versus Tufi[b]	6.49	0.68	Palau[c]	2.21
Nausithoe punctata					
	–	–	–	Palau[d]	8.99
Nausithoe racemosa					
	Palau versus Observation Point	0.18	0.21	Palau	0.00
	Palau versus Duchess Island	0.00	0.00	Observation Pt.	0.37
	Observation Point versus Duchess Is.	0.18	0.21	Duchess Island	0.00
Periphylla periphylla					
	Norway versus Malvinas	0.00	0.00	Norway	0.00
Physalia physalis					
	Australia versus Kauai[e]	1.28	1.21	Australia	1.72

[a] Mean PSD, s.d. = 0.18, number of individuals (n_i) = 3.
[b] Partial sequence.
[c] Mean PSD, s.d. = 1.17, n_i = 3.
[d] Mean PSD, s.d. = 6.20, n_i = 5.
[e] Approximate latitudes and longitudes of sites are: Alaska (Kachemak Bay: 59° 30′ N 151° 50′ W), California (Morro Bay: 35° 22′ N 120° 52′ W), South Africa (Hout Bay: 34° 01′ S 18° 21′ E), Emona (10° 01′ S 150° 57′ E), Observation Point (09° 44′ S 150° 44′ E), Norway (Raunefjorden: 60° 13′ N 5° 16′ E), Australia (Merimbula Lake: 36° 54′ S 149° 54′ E; Huon Estuary: 43° 17′ S 147° 07′ E), Palau (Risong Cove: 07° 16′ N 134° 31′ E), Tufi (09° 05′ S 149° 19′ E), Duchess Island (09° 58′ S 150° 51′ E), Malvinas (52° 00′ S 59° 30′ W), Kauai (22° 00′ N 159° 41′ W).

while Medusozoa (Petersen, 1979) would more intuitively be applied to only the tesserazoan clades containing free-living medusae, i.e. excluding stauromedusae (now considered Class Staurozoa: Marques & Collins, 2004). Cubozoa and Hydrozoa would remain valid classes in a phylogenetic scheme. Semaeostomeae, which historically refers to an evolutionary group that includes rhizostomes (e.g. Mayer, 1910), but in its usual sense excludes them, would be valid in its broader sense and with Coronatae would form a monophyletic Class Scyphozoa (Collins, 2002). Alternatively, it may be useful to retain Semaeostomeae as a term exclusive of rhizostomes, in which case Discomedusae may best describe semaeostomes-plus-rhizostomes (e.g. *sensu* Maas, 1907; Naumov, 1961; M.N. Arai, pers. commun.) although, in some early works the term also included Coronatae (e.g. Haeckel, 1879; Bigelow, 1904). The term Discophora, also used for semaeostomes-plus-rhizostomes and sometimes including coronates (e.g. Bigelow, 1909), is less intuitive and has also been applied to several non-cnidarian taxa.

Molecular analyses suggest current nomenclature may also poorly represent some relationships at lower taxonomic levels. For example, *Nausithoe* may be polyphyletic (Fig. 1), but discrepancies are most numerous at the species level.

Pairwise comparisons within-morphospecies-between-regions and within-morphospecies-within-regions reveal four groups (two *Cyanea capillata* in Australia, *Mastigias papua*, *Nausithoe punctata*) distinguished from 'conspecifics' by sequence distances of ≥6.5%, which indicates possible species-level differences (Dawson & Jacobs, 2001). 'Scyphozoan' species diversity may be about twice recent estimates based on morphology (Mianzan & Cornelius, 1999), irrespective of species yet to be described (e.g. Lindsay et al., 2004), based on molecular analyses of 13 scyphozoan morphospecies thus far sampled from more than one region (*Aurelia aurita*, *A. labiata*, *A. limbata*, *Cassiopea andromeda*, *C. frondosa*, *C. xamachana*, and those in Table 2) that indicate the existence of approximately 15 cryptic species (Table 2; Dawson, 2003; B.S. Holland et al., in press). The same may be true of Hydrozoa, in which preliminary molecular analyses of *Aequorea aequorea* indicate cryptic species (see also Schuchert, 1998).

Morphological evolution

Cryptic species may be morphologically differentiable (Knowlton, 2000), as may populations (Dawson, 2003), which opens up a range of research opportunities. Investigations of geographic variation and temporal variation – such as morphological transformations that occur at speciation – become tractable. Set in an appropriate evolutionary context, such studies can find evidence for, among other mechanisms, selection, drift, and trade-offs with behaviour (Dawson, 2005).

The molecular phylogeny of medusae also elucidates morphological differences at higher taxonomic levels and suggests long-standing inconsistencies in the naming of radial canals, which are key taxonomic features (Stiasny, 1921). Mayer (1910) referred to the area between adjacent gastric pouches as 'interradial' in rhizostomes but 'perradial' in semaeostomes, although he considered the cruciform stomach of rhizostomes and gastric pouches of semaeostomes to be homologous. Similarly, Uchida (1926) placed interradial canals in *Mastigias papua* and perradial canals in *Aurelia* in the same position, relative to the gastric pouches, although he considered interradial canals in rhizostomes and semaeostomes homologous (see also Russell, 1970). This implies the canal system rotated 45° relative to the gastric pouches during the evolution of rhizostomes from semaeostomes or a semaeostome-like ancestor. However, the criteria of 'correspondence of position' and 'transitional forms' (see Futuyma, 1998) both suggest a simpler interpretation – that canals originating at the distal terminus of each gastric pouch are homologous but named differently in rhizostomes and semaeostomes. Probably, these canals are best termed 'interradial,' as they are currently in semaeostomes on the basis of their positions relative to the perradial mouth lips (Russell, 1970, p. 3), although the gastric pouches, which are easily identified throughout the development of rhizostome and semaeostome medusae, may be better points of reference. Additional developmental work may be needed to resolve this issue fully.

Behavioural evolution

Behavioural variation, like morphological variation, is widely known (Arai, 1997) but most is poorly documented in medusae. Relatively well-known variations in diel vertical migration and prey selection often have been attributed to ontogenetic and environmental factors or ascribed to intra-specific variability and interpreted as evidence of phenotypic plasticity. However, the plethora of cryptic species indicated by molecular analyses now suggests that, like morphological variation, at least some behavioural differences represent heritable local variations rather than ecological generalism and plasticity (Dawson & Martin, 2001). Such variation may reflect underlying physiological differences (e.g. McCloskey et al., 1994) resulting from an array of evolutionary processes, including adaptation (Dawson & Hamner, 2003).

Exchangeability, biodiversity management, and species

Adaptation is a key consideration in conservation biology. For almost a decade, conservation biologists focused on genetic analyses of neutral markers, assuming they were good indicators of evolutionarily significant units (e.g. Moritz, 1994). However, recent analyses have indicated that neutral markers may not reflect immunological and other kinds of adaptation and, consequently, the broader concept of ecological equivalence or 'exchangeability' (Templeton, 1989) has received renewed attention (Crandall et al., 2000; see also the 'symmetry' concept of Stewart, 2003). This concept is particularly illuminating when considering anthropogenic translocations, which threaten biodiversity worldwide (Bax et al., 2001). For example, when *Aurelia aurita* was considered as a circumglobal, generalist species, it was of little concern if individuals were translocated via ballast water or hull fouling. However, now that molecular, behavioural, morphological, and physiological data indicate there are at least a dozen geographically restricted and likely locally adapted species of *Aurelia*, unusually wide distributions due to translocations are appropriately recognized as the result of introductions (Dawson, 2003). This, in turn, raises awareness of the potential ecological impacts of these voracious predators, which can increase rapidly in number and size (Mills, 2001), and the need to prevent or mitigate introductions.

That jellyfishes are more geographically restricted and evolutionarily diverse also has commercial implications. Rhizostomes include at least 11 commercially harvested food species (Omori & Nakano, 2001). If these species are composites of subdivided stocks, as indicated for *Catostylus mosaicus* by Pitt & Kingsford (2000), then management strategies should reflect stock structure to reduce the chance of over-exploitation.

The idea of exchangeability also applies to species-level biodiversity management, as it is central to the cohesion species concept (CSC: Templeton, 1989). As with all species concepts, there are problems of applicability in certain cases and of determining precisely what differences merit species-level recognition, but a schema has been proposed to guide interpretation of variation in terms of both conservation and speciation (Crandall et al., 2000). The CSC fosters an holistic perspective of biodiversity by promoting explicit consideration of present and past variation in the manifold attributes of organisms – including behaviour, molecules, morphology, and physiology – that influence their ecology and distribution. Applying the CSC should also, therefore, promote continuity between traditional morphological taxonomy and more recent molecular phylogenetic approaches, a desirable integration that is inhibited by the misconception that cryptic species are morphologically identical.

Future directions

Clearer relationships among classes and lower taxonomic levels of cnidarians enable richer evolutionary studies, increase our understanding of patterns of diversity in the Cnidaria, and elucidate patterns and processes of evolution in the Metazoa and marine environment. Although relationships among orders and classes of cnidarians are still relatively poorly resolved, the evolution of polyp and medusa forms is becoming clearer. The basal position of Anthozoa within Cnidaria is now widely accepted (Bridge et al., 1992, 1995). Most molecular data now strongly suggest that staurozoans, a modified polyp form, are the most basal

tesserazoans. Thus, the medusa evolved as a secondary life-stage and may be synapomorphic for medusozoans (Hydrozoa, Cubozoa, Coronatae, and Discomedusae; but see Salvini-Plawen, 1987). In this context, it is simple to postulate that the medusa evolved as a dispersal stage, but other advantages such as access to planktonic food resources, rapid growth, and increased reproductive potential may also be involved. As has long been recognized (Collins, 2002), the evolution of the polyp, the nature of the polyp–medusa metamorphosis, and the loss, regain, and specialization of both life-stages provide a rich context for discussing life-history evolution.

The influence of life history on genetic structure has received considerable attention in the marine environment. Popular hypotheses link longer pelagic larval duration, more offshore (open) habitats, and higher fecundity to lower genetic structure in a variety of generally sexually reproducing organisms with planktonic larval but benthic juvenile and adult stages, including reef fishes, molluscs, echinoderms, and sea anemones (e.g. Waples, 1987; Hellberg, 1996; Murray-Jones & Ayre, 1997). Studies of medusozoans additionally allow the effects of bipartite life-histories and asexual reproduction to be investigated in organisms in which the sexually reproducing form is typically the dispersive phase. The independent loss and gain of polyp or medusa in diverse medusozoans permits the effect of different strategies to be tested multiple times, leading to robust general conclusions about links between life-history, geographic structure, and patterns of diversity that are influenced little by historical contingencies.

Preliminary data are sparse but highly suggestive. For example, coastal taxa with benthic asexually reproducing polyps (*Aurelia*, *Cassiopea*, *Mastigias*) comprise many geographically restricted species with deep evolutionary histories (Dawson & Jacobs, 2001; Holland et al., in press; Dawson, in press; but see *N. racemosa*), whereas oceanic sexually reproducing holoplanktonic species lacking a benthic phase have haplotypes common to northern and southern Pacific (*Physalia physalis*) and Atlantic (*Periphylla periphylla*) oceans. Hypothetically, populations occurring in extreme isolation due to habitat (e.g. *Mastigias*) or life-history (e.g. the almost holobenthic *Cassiopea*) will show the greatest geographic structure, potentially linked to environmental variation (e.g. Dawson & Hamner, 2003). Populations of such restricted species may, therefore, be good candidates for examining ecological specialization and speciation. In this context, rhizostomes are additionally interesting because they are the most diverse and highly derived scyphozoans (Mayer, 1910; Collins, 2002), are largely restricted to the Indo-West Pacific centre of marine biodiversity (Mianzan & Cornelius, 1999). An accurate systematics of Rhizostomeae, placed appropriately within Scyphozoa, will facilitate studies with broad interest. For example, is diversity in Rhizostomeae consistent with accumulation, origin, or overlap of species in the Indo-West Pacific? Is diversity contributed to by invasion of ecological niches such as photosymbioses, which are prevalent in tropical rhizostomes? Is the high morphological diversity of rhizostomes, as indicated by the large number of taxa at each level of the Linnean hierarchy, matched by high molecular diversity (relative to other scyphozoans)?

Concluding remarks

Medusae are one of the most successful groups of marine organisms, arguably predating the Cambrian explosion. They are interesting in terms of the evolution of body plans, development, and life histories because of their deep phylogenetic origin somewhere – it is unclear precisely where (e.g. Medina et al., 2001) – amongst the origins of the Metazoa, bilateria, and triplobasts. An accurate phylogeny for the Tesserazoa will allow an informed choice of model organisms and provide realistic evolutionary scenarios for investigating high-profile topics such as the evolution of eyes (Martin, 2004), clonality, coloniality, asexual and sexual reproduction, and polyp–medusa life cycles (Collins, 2002). Comparison of medusae and polyp stages should also illuminate the expression of two different organisms from a single genome. In addition, the documentation of morphological differences among populations of *Mastigias* whose evolutionary history is well understood and polarised offers the chance to study links between development and the early stages of speciation. Such endeavours will benefit from the application

of the broad range of molecular techniques that are becoming available to studies of a variety of non-model organisms. Advances in technology, large-scale sequencing projects, the electronic rejuvination of old data, and techniques for managing new, large, datasets should soon result in dramatic advances in cnidarian biology and many related disciplines.

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On the taxonomy and presumable evolutionary pathways of planktonic larvae of Ceriantharia (Anthozoa, Cnidaria)

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Key words: Arachnactidae, cnidom, paedogenesis

Abstract

After investigation of the type specimens of 11 genera of ceriantharian larvae, it was concluded that the overwhelming majority of planktonic forms should be referred to the family Arachnactidae. Despite the fact that the presence of acontioids is traditionally considered as the only characteristic feature of this family, in most of the species acontioids develop at rather late ontogenetic stages. The importance of another feature, the presence or absence of *p*-rhabdoids, was considered. Members of suborder Penicillaria (Arachnactidae) typically have *p*-rhabdoids in the endoderm of alternate mesenteries and in acontioids. Members of suborder Spirularia (Cerianthidae and Botrucnidiferidae) have no *p*-rhabdoids and all cnidae in their mesenteries are restricted to the region of the mesenteric filament. In Arachnactidae the number of known larval forms significantly exceeds the number of benthic ones. The planktonic forms with developing and mature gonads were described in this family. The paedogenetic origin of Arachnactidae is discussed. It seems likely that, due to the uniformity and deficiency of definite morphological characteristics of the benthic members of the family Arachnactidae, only cnidom and type of the planktonic larva can be used for reliable taxonomical identification of the polyps.

Introduction

Speaking about Ceriantharia we usually bear in mind solitary, anemone-like anthozoans with two rings of tentacles, which inhabit fibrous mucous tubes. Ceriantharia are known from sedimentary substrata at SCUBA diving depths in seas throughout the world. In some biotopes cerianthids can be dominant species – as *Cerianthus lloydii* Gosse, 1859, at shallow-water hydrothermal vents of Kraternaya Bight (Kurile Isles). This species reaches population densities up to several hundreds m^{-2} (Molodtsova & Malakhov, 1995).

Sars (1846) described from Florø Island a species of floating actinian, *Arachnactis albida*, possessing two rings of tentacles. The name *Arachnactis* was chosen because of the general appearance that resembled a spider, due to its widely extended outer tentacles and tiny inner ones. Afterwards this species was considered a planktonic larva of some ceriantharian, but until now the benthic form of this larva still remains unknown. Larval development has been studied in only some ceriantharians. It was shown (Carlgren, 1906) that such species as *Cerianthus lloydii* Gosse, *C. borealis* Verrill, and *C. membranaceus* (Spallanzani) possess long-living planktonic larvae. For *Pachycerianthus multiplicatus* (Carlgren, 1912 a, b), and *P. magnus* (Nakamoto, 1919), short-living demersal larvae were reported (Nyholm, 1943; Uchida, 1979). For *C. misakiensis* (Nakamoto, 1923), early planulae were described (Song, 1986) but the further development was not traced. Plenty of larval forms were described from the plankton of tropical and subtropical areas of the ocean (van Beneden, 1897, 1923; Carlgren, 1924b; Leloup,

1932, 1942, 1964, 1968). Planktonic larvae of Ceriantharia have been described under their own binomens and from the end of the 19th century through to end of the 20th century about 90 species belonging to 32 genera were recognized. For comparison, so far only 50 valid species belonging to eight genera of benthic Ceriantharia have been described. In some ceriantharian larvae gonad formation was reported and, in view of this, the paedogenesis hypothesis was intensively discussed (Vanhöffen, 1895; Bourne, 1919).

Genera of ceriantharian larvae are referred to the same families as the benthic forms: Cerianthidae, Bortrucnidiferidae, and Arachnactidae.

Den Hartog (1977) proposed two suborders in the order Ceriantharia: Spirularia (combining Cerianthidae and Botrucnidiferidae) and Penicillaria (including Arachnactidae). He found that besides the anatomical features (length of hyposulcus, presence of derivatives of mesenterial filament, and arrangement of mesenteries), there is a clear difference in the cnidom between these two groups (Table 1). Only in the cnidom of arachnactids were *p*-rhabdoids (penicillae in nomenclature of Stephenson, used by den Hartog) reported.

The main purpose of this work was to study ceriantharian larvae to establish how the features proposed by den Hartog (1977) are distributed between planktonic forms of the three families of Ceriantharia.

Material and methods

In October–December 1999 due to the kind assistance of curators of the collections, I had an opportunity to study the type specimens of ceriantharian larvae housed in the Zoological Museum of the University of Liège (Liège, Belgium) (16 species of 11 genera described by van Beneden and Cerfontain) and the Museum of Natural Sciences (Brussels, Belgium) (nine species of nine genera described by Leloup). The material studied was represented by the original series of microscope slides. Species of all three families of Ceriantharia were examined (genera *Bursanthus, Nautanthus, Sacculactis, Apiactis, Solasteractis, Peponactis, Synarachnactis, Syndactylactis, Isodactylactis, Calpanthula, Angianthula, Cerianthula, Hensenanthula, Sphaeranthula, Arachnactis, Dactylactis, Ovactis, Isarachnactis* and *Isapiactis*). Additionally I examined the microscope slides of the type specimen of the benthic arachnactid *Arachnanthus oligopodus* (Cerfontaine, 1891a, b), also housed in the Zoological Museum of the University of Liège.

The nomenclature of cnidae used is that of Schmidt (1974).

Results

p-Rhabdoids were present in all arachnactid larvae studied. In the species of the genera *Isarachnactis* (Carlgren, 1924a, b), *Dactylactis* (van Beneden, 1897), *Isapiactis* (Carlgren, 1924a, b), *Arachnactis* (M. Sars, 1846), and *Ovactis* (van Beneden, 1897) numerous *p*-rhabdoids were found not only in ectoderm of the oral disk and tentacles but also in the endoderm of mesenteries as well as in the endoderm of the body wall. Generally *p*-rhabdoids found in endoderm were concentrated in the lower

Table 1. Suborders of Ceriantharia

Suborder Spirularia		Suborder Penicillaria
Cerianthidae	Botrucnidiferidae	Arachnactidae
p-Rhabdoids absent		*p*-Rhabdoids present
Homotrichs present in the column		Homotrichs absent in the column
Mesenteries in quartets		Mesenteries in duplets
Short hyposulcus		Long hyposulcus often bordered with ciliated tract
Acontioids absent		Acontioids present
Craspedonemes often present		Craspedonemes absent
Cnidorages present or absent		Cnidorages absent

region of alternate mesenteries and gastral cavity. However, in *Dactylactis armata* (van Beneden, 1897), and *D. digitata* (van Beneden, 1897), numerous *p*-rhabdoids were also situated in the endoderm of tentacles. The same type of cnidae distribution was found in the Mediterranean benthic arachnactid *Arachnanthus oligopodus* (Cerfontaine, 1891a). Here *p*-rhabdoids were observed in the endoderm of alternate mesenteries at the level of acontioids.

Other features shared by all Arachnactidae include the broad ciliated tract of alternate mesenteries (type 1 of Carlgren, 1912a), wide siphonoglygh, and well-developed hyposulcus, often provided with ciliated streaks on its exocoelic sides.

On the other hand, examination of the type specimens of planktonic larvae of *Sacculactis guntheri* (Leloup, 1964), and *Bursanthus bamfordi* (Leloup, 1968), has revealed that the structure of the ciliated tract of alternate mesenteries and hyposulcus, as well as the presence of numerous large *p*-rhabdoids in the endoderm of alternate mesenteries and column wall, make these species closer to arachnactid than to cerianthid larvae studied.

From the results obtained it is possible to see that the presence and absence of *p*-rhabdoids is a feature which correlates with the general morphological features generally used in taxonomy of Ceriantharia (McMurrich, 1910; Carlgren, 1912a, b; den Hartog, 1977), such as the structure and arrangement of the mesenteries and the length of hyposulcus, not only in benthic species but also in long-living planktonic larvae. It was found also that in all Arachnactidae studied, *p*-rhabdoids were present in endoderm of lower part of column wall and alternate mesenteries, often close to acontioids. This feature is also known in benthic *Arachnanthus oligopodus*. *p*-Rhabdoids were also found in the endoderm of lower part of gastral cavity of *Isarachnanthus maderensis* (Johnson, 1861), common in the Central Atlantic and Caribbean region (Molodtsova, 2003).

Unfortunately I was not able to study species of *Anactinia* (Annandale, 1909), *Paranactinia*, (Carlgren, 1924a, b) and *Isovactis* (Leloup, 1942), to make an exhaustive account on the presence of *p*-rhabdoids in the endoderm of the members of this family.

On the other hand, it is necessary to take into account that in the well-known arachnactid *Arachnactis albida* the acontioids develop only at a stage with nine marginal tentacles and 16 mesenteries (Bourne, 1919), and in *Anactinia pelagica* the number and arrangement of acontioids are often inconstant (Annandale, 1909). Many species of ceriantharian larvae are known at much earlier stages of development. Even in benthic arachnactids the arrangement of acontioids can vary. Thus in *Isarachnanthus maderensis* (Brito, 1986) acontioids at the second pair of protomesenteries are often absent and in *I. panamensis* some specimens with no trace of acontioids were reported (Carlgren, 1924a). In view of the aforesaid facts, it is hard to expect that the presence and absence of acontioids could be considered as the main characteristic feature of the family, especially in the case of planktonic larvae. It appears that such features as the presence of *p*-rhabdoids in the endoderm of alternate mesenteries, a wide siphonoglyph with several pairs of mesenteries attached, a long hyposulcus with ciliated streaks on its exocoelic sides, and a broad ciliated tract of alternate mesenteries are much more characteristic. Therefore the species *Sacculactis guntheri* (Leloup, 1964) and *Bursanthus bamfordi* (Leloup, 1968) should be considered as members of Arachnactidae. Also, based on the original descriptions, it can be suggested that *Nautanthus bathypelagicus* (Leloup, 1964), *Parovactis clavata* (Leloup, 1964), and *Engodactylactis formosa* (Gravier, 1920) belong to the Arachnactidae (Table 2).

Thus, I propose the following diagnosis of the suborder Penicillaria: Ceriantharia with *p*-rhabdoids in ectoderm and *p*-rhabdoids occurring in ectoderm and endoderm of alternate mesenteries and column wall. Mesenteries in benthic forms arranged in doublets. Stomodeum with wide siphonoglyph and long hyposulcus ($\approx$0.5–1.0$\times$ the length of the stomodeum proper), bordered by ciliated streak at exocoelar sides. Craspedonemes and cnidorages always absent. Acontioids often present but could be absent in planktonic larvae and rare in benthic forms. One family, Arachnactidae, with diagnosis of the suborder.

One of the most remarkable facts in Ceriantharia biology is the existence of mature or developing gonads in several species of planktonic

Table 2. Cerianthid taxonomy after Carlgren (1912a, 1924a, b, 1931); Leloup (1964, 1968) (modified)

	Cerianthidae	Botrucnidiferidae	Arachnactidae
Benthic forms	*Cerianthus*	*Botruanthus*	*Arachnanthus*
	Pachycerianthus	*Botrucnidifer*	*Isarachnanthus*
	Ceriantheopsis		
	Ceriantheomorphe		
Planktonic forms	*Anthoactis*	*Angianthula*	*Anactinia*
	Apiactis	*Atractanthula*	*Arachnactis*
	Isodactylactis	*Botrucnidiata*	*Bursanthus*
	Paradactylactis	*Calpanthula*	*Dactylactis*
	Peponactis	*Cerianthula*	*Engodactylactis*
	Plesiodactylactis	*Gymnanthula*	*Isapiactis*
	Solasteractis	*Hensenanthula*	*Isarachnactis*
	Synarachnactis	*Ovanthula*	*Isovactis*
	Syndactylactis	*Sphaeranthula*	*Nautanthus*
	Trichactis		*Ovactis*
			Paranactinia
			Parovactis
			Sacculactis

Table 3. Matured and developing gonads in planktonic larvae of Ceriantharia

Species	Records	Stage of gonad development	Family
Anthoactis armauerhanseni	Leloup (1932)	Oocytes	Cerianthidae
Arachnactis albida	Bourne (1919)	Matured gonads	Arachnactidae
	Vanhöffen (1895)		
Anactinia pelagica	Annandale (1909)	Oocytes	Arachnactidae
Dactylactis armata	van Beneden (1897)	Oocytes	Arachnactidae
Dactylactis benedeni	Gravier (1904)	Oocytes	Arachnactidae
Dactylactis brachysoma	Cerfontaine (1891b)	Oocytes	Arachnactidae
Engodactylactis formosa	Gravier (1920)	Oocytes	Arachnactidae
Nautanthus bathypelagicus	Leloup (1964)	Matured gonads	Arachnactidae
Ovactis brasiliensis	van Beneden (1897)	Oocytes	Arachnactidae

larvae (summarized in Table 3). It has to be emphasized that eight of nine reported cases the developing gonads were observed in species of Arachnactidae. The only two known cases of mature gonads in planktonic Ceriantharia (*Arachnanthus albida* and *Nautanthus bathypelagicus*) were also reported in Arachnactidae. I was not able to study the type material of *Anthoactis armauerhanseni*; however, some morphological features (the length of hyposulcus and the structure of ciliated tract of alternate mesenteries) of the species included in this genus could be evidence for questioning the position of this genus.

Since the discovery of gonads in ceriantharian larvae, the hypothesis of paedogenesis has repeatedly been discussed with respect to two families of the order Ceriantharia – Arachnactidae (Bourne, 1919; Leloup, 1954, 1964) and Botrucnidiferidae (van Beneden, 1897; Carlgren, 1924b). In my view the paedogenetic origin of the family Arachnactidae could be supported by the following facts: (1) the number of larval forms described significantly exceeds the number of benthic ones (33 species of larvae and seven species of benthic polyps), (2) the majority of planktonic forms with developing and mature gonads (eight of nine reported) were de-

scribed in the family Arachnactidae, and (3) the characteristic morphology of adult arachnactids (few tentacles and mesenteries) makes them resemble enormously enlarged larvae of *Arachnactis* or *Isarachnactis*. Apparently, the long hyposulcus, broad siphonoglyph, and structure of the ciliated tract of alternate mesenteries, as well as large *p*-rhabdoids in the endoderm, were developed in Arachactidae as an adaptation to a long life in the plankton. It is not inconceivable that at least some Arachnactidae could represent exclusively planktonic animals having a life cycle of 1 year.

It is concluded that, due to uniformity in appearance and morphology of benthic Arachnactidae, only the cnidom and the type of planktonic larva can serve as reliable features for taxonomical identification of adult arachnactids.

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Cloning and sequencing of cDNA encoding glutamine synthetase from the sea anemone *Aiptasia pallida*

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Abstract

Glutamine synthetase (GS) catalyzes the addition of ammonium to glutamic acid to form glutamine and plays a crucial role in the nitrogen assimilation of the sea anemone *Aiptasia pallida* and its endosymbiotic algae. We describe the cDNA cloning and sequence analysis of GS mRNA from *A. pallida* based on polymerase chain reaction (PCR) technology that employed a combination of degenerate and *A. pallida*-specific primers. The sequenced cDNA approximates 1620 nucleotides and is characterized by an open reading frame of 1107 nucleotides that encodes a protein of 369 amino acid residues. Comparisons of the deduced sea anemone GS protein to a wide range of species demonstrated greatest amino acid sequence identity to sea urchin GS (66%) and least identity to green algae GS (51%). The sequenced cDNA can be used in future research to study GS gene expression in *A. pallida*.

Introduction

The ability of algal-invertebrate symbioses to assimilate and retain nitrogen from N-poor environments is thought to play an important part in their evolutionary success. However, the role that each organism plays in the uptake, assimilation, and retention of inorganic nitrogen has not been completely resolved and may vary from one symbiosis to another and with the nitrogen status of each association.

Tracer studies employing ^{15}N-ammonium as a nitrogen source provide the strongest evidence for the role of each partner in the assimilation of inorganic N. In assimilation studies using the scyphozoan, *Linuche unguiculata* (see Wilkerson & Kremer, 1992), the giant clam, *Tridacna gigas* (see Hawkins & Klumpp, 1995), the scleractinian coral, *Stylophora pistillata* (see Grover et al., 2002), and the temperate anemone, *Anemonia viridis* (see Roberts et al., 1999), zooxanthellae appear to be the primary site of nitrogen assimilation. However, some tropical cnidarian hosts are also capable of direct incorporation of ammonium. Lipschultz and Cook (2002) found that the zooxanthellate tentacles and the azooxanthellate body column of *Bartholomea annulata* incorporated ^{15}N-ammonium. Likewise, host tissues of zooxanthellate and aposymbiotic *Aiptasia pallida* assimilated inorganic N. In both species of anemones, animal ammonium assimilation was greater when endosymbiotic algae were present.

Despite the differences in these studies, it is generally accepted that glutamine synthetase (GS) is the primary enzyme responsible for inorganic N incorporation into algal-invertebrate symbioses. This enzyme catalyzes the conversion of ammonia and glutamate to the amide, glutamine, with the simultaneous hydrolysis of ATP. It has long been known that microalgae assimilate ammonia using GS (Syrett, 1981). More recently the presence of GS in host tissues has been documented in *Trid-*

acna gigas (see Rees et al., 1994), *Pocillopora damicornis* (see Yellowlees et al., 1994), *Aiptasia pallida* (see Ferrier et al., 1996), and *A. pulchella* (see Wang & Douglas, 1998).

The direct incorporation of ^{15}N-ammonium into *A. pallida* (Lipschultz & Cook, 2002) is supported by the findings of Ferrier et al. (1996) that anemone tissue from both zooxanthellate and aposymbiotic *A. pallida* possessed GS activity. Furthermore, host GS activity was positively correlated with algal density and further enhanced in unfed zooxanthellate *A. pallida* maintained in low-nutrient seawater (Ferrier et al., 1996).

The purpose of this study is to report the cloning and sequence analysis of GS cDNA from unfed *A. pallida*. The availability of cDNA will facilitate future studies of GS gene expression in *A. pallida*, including RNA characterization and quantitation under a range of nutrient conditions.

Methods and materials

Animal culture and RNA extraction

Clonal *Aiptasia pallida* originally collected from Walsingham Pond, Bermuda, were maintained in glass bowls containing 30 ppt artificial seawater at 25°C and a photoperiod of 12 h light:12 h dark (Muller-Parker et al., 1990). Animals were fed twice weekly with freshly hatched brine shrimp, *Artemia* sp., and their seawater changed 24 h after feeding. Prior to RNA isolation, animals were not fed for a period of 2 week and the seawater was changed twice weekly.

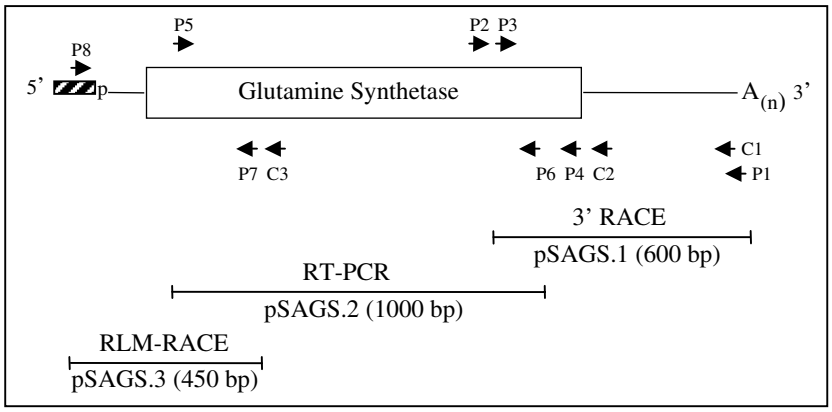
Total RNA was isolated from anemones by gently homogenizing two-four animals (100 mg) for 5 min in a glass manual tissue grinder on ice in 5 volumes (0.5 ml) of buffer containing 10 mM Tris (pH 7.0) and 560 mM NaCl. The homogenate was transferred to 1.5-ml microcentrifuge tubes and centrifuged at 4°C for 1 min at $12\,000 \times g$ to remove algal cells and anemone tissue debris. Following a second centrifugation to remove residual algal cells and tissue debris, RNA was extracted from the supernatant by the method of Chomczynski (1993) using Trizol LS Reagent (Invitrogen, Inc., Carlsbad, CA). Isolated RNA was treated with DNase I, Amplification Grade (Invitrogen, Inc.) to remove contaminating genomic DNA. The concentration of RNA was determined spectrophotometrically and its integrity was verified by the presence of intact ribosomal RNA on a 1.0% agarose gel stained with ethidium bromide (Sambrook & Russel, 2001).

Cloning and sequencing of A. pallida *GS cDNA*

Reverse transcription (RT) and polymerase chain reaction (PCR) technology (Dieffenbach & Dveksler, 1995; Sambrook & Russel, 2001) were used to produce GS cDNA, including protocols for the rapid amplification of cDNA ends (RACE) and RNA-ligase-mediated (RLM) RACE (Schaeffer, 1995). Figure 1 summarizes our overall strategy to produce GS cDNA using these PCR-based protocols. Briefly, GS sequences from Genbank for yeast (M65157), nematode (Z29560), and fly (X52579) were used to design degenerate primers to produce a 3′ cDNA employing a 3′ RACE kit (Invitrogen, Inc.). The 600-bp amplification product was cloned into pGEM-3Z (Promega Corp., Madison, WI) and sequenced using an ABI Prism 310 DNA sequencer (Applied Biosystems, Foster City, CA). The resulting data from two independent cDNA clones (pSAGS.1) were used to design gene-specific primers for RT-PCR in combination with a degenerate primer designed from yeast, nematode, and fly. The resulting RT-PCR product (1000 bp) was cloned into pCRII-TOPO (Invitrogen, Inc.), and the complete sequence analysis of two independent clones was conducted to yield the central region of GS (pSAGS.2). Sequencing data obtained from pSAGS.2 were used to design primers to amplify the 5′ end of the gene by means of RLM-RACE employing a GeneRacer kit (Invitrogen, Inc.). The RLM-RACE product of 450-bp was cloned into pCR4-TOPO to produce multiple clones of the 5′ portion of GS for sequence analysis.

The GCG software package (Womble, 2000) was used for analysis of sequence data, including the programs GAP, MAP, PEPTIDESORT, and TRANSLATE. The analysis of multiple GS protein sequences was conducted using the GCG programs PILEUP, PAUPSEARCH, and PAUP-DISPLAY and employed a maximum parsimony method to estimate phylogenetic relationships with a bootstrap replication of 100.

A



B

	RLM-RACE	RT-PCR	3' RACE
Primer for cDNA synthesis	C3	C2	C1
Primers for PCR	P7 and P8	P4 and P5	P1 and P2
Primers for nested PCR	None	P5 and P6	P1 and P3
Product (cDNA) size	450 bp	1000 bp	600 bp

C

Primer	Sequence (5' to 3')	Source
C1	ggccacgcgtcgactagtacttttttttttttttt	3' RACE Kit (Invitrogen, Inc.)
C2	tgaacagaaacagcagtactcc	pSA GS.1
C3	atacaggtctttctgttgttgg	pSA GS.2
P1	ggccacgcgtcgactagtac	3' RACE Kit (Invitrogen, Inc.)
P2*	gg(a,t,c)ga(c,t)tggaacgg(a,t,c)gc(c,t)gg	yeast, nematode, fly
P3*	cccaagctt(c,t)(a,t)(c,g)i(t,g)(c,g)igg(a,t,c)gt(a,c,g)gccaa	yeast, nematode, fly
P3	tgaacagaaacagcagtactcc	pSA GS.1
P4	gatcacagtttgatgatggtcg	pSA GS.1
P5*	gt(g,t)tgg(a,g)tcga(t,c)gg(c,a,t)(a,c)c(c,t)gg	yeast, nematode, fly
P6	caagataacccttgccatcc	pSA GS.1
P7	ggtcgtgtgtgtaggtctcgcag	pSA GS.2
P8	cgactggagcacgaggacactga	Gene Racer Kit (Invitrogen, Inc.)

*P2, P3, and P5 are degenerate primers based on conserved amino acid sequences determined by PILEUP analysis of glutamine synthetase sequences for yeast, nematode, and fly. The Genbank accession numbers for these organisms are listed in Figure 3.

Figure 1. Summary of the PCR-based strategy to produce glutamine synthetase cDNA using RNA extracted from the sea anemone *Aiptasia pallida*. (A) Location of primers employed to generate and amplify cDNA employing 3′ RACE, RT-PCR, and RLM-RACE protocols. The hatched box symbolizes an RNA oligonucleotide that was ligated to the 5′ end of mRNA for the RLM-RACE protocol. (B) Specific primers used for each protocol and size of the final cDNA product. (C) Sequence information for primers used in (A) and (B).

Results and discussion

Our PCR-based strategy for the isolation of cDNA corresponding to GS mRNA from the sea anemone *A. pallida* resulted in the production of three cDNA clones that form an overlapping representation of the mRNA. As shown in Figure 1A, these plasmid clones designated pSAGS.1 (3′ region), pSAGS.2 (central region), and pSAGS.3 (5′ region) contain cDNAs generated by 3′ RACE (600-bp product), conventional RT-PCR (1000-bp product), and RLM-RACE (450-bp product), respectively. Our 3′ RACE procedure was based on the use of degenerate primers designed from conserved amino acids determined for yeast, nematode, and fly. Prior to cDNA cloning,

agarose gel electrophoresis of these 3′ RACE products revealed two major cDNA products of 600 and 950 bp (data not shown). Subsequent sequence analysis of four cDNA clones representing each of these size products revealed that two of the 600-bp cDNA clones shared 72% amino acid identity to the carboxy-terminal portion of GS from *Drosophila melanogaster* (Caizzi et al., 1990). As summarized in Figure 1, following sequence analysis of the 3′ cDNA (pSAGS.1), we completed the cDNA cloning of GS in two parts by using RT-PCR to produce cDNA to the central region of GS mRNA (pSAGS.2), followed by RLM-RACE to yield cDNA (pSAGS.3) that corresponds to the 5′ region of the mRNA.

The complete nucleotide sequence of glutamine synthetase is presented in Figure 2. The open reading frame is characterized by 1107 nucleotides and encodes a protein of 369 amino acids with a calculated molecular mass of 41.7 kDa. The 5′

```
   1  attcctctatttattcagacttgatatttgtagtattccccgtcgttattagtaagctaa
  61  atttaaggacagagcc**atg**agtcttcacgataaaagaactgtctctgacaagacatgtgt
                      M  S  L  H  D  K  R  T  V  S  D  K  T  C  V    15
 121  tttggagaggtattcgaaacttgaccaaggaaccagcgttcaagccttgtacatttggat
       L  E  R  Y  S  K  L  D  Q  G  T  S  V  Q  A  L  Y  I  W  I    35
 181  tgacggtacaggcgaaggtatccgttgtaaaaccaagactttagagaaaaggccagattc
       D  G  T  G  E  G  I  R  C  K  T  K  T  L  E  K  R  P  D  S    55
 241  cattgaagaccttccaatctggaacttcgatggatcgagtaccgcacaagccgagggcca
       I  E  D  L  P  I  W  N  F  D  G  S  S  T  A  Q  A  E  G  H    75
 301  taacagcgatgtctatcttcatcctgtagctattttcaaggacccattccgtggcggaga
       N  S  D  V  Y  L  H  P  V  A  I  F  K  D  P  F  R  G  G  E    95
 361  aaacatcttggttctctgcgagacctacacacacgaccataagccaacacacaccaacaa
       N  I  L  V  L  C  E  T  Y  T  H  D  H  K  P  T  H  T  N  N   115
 421  cagaaagacctgtatgaacataatgaacgaccctgaagttaaatcttcacatccatggtt
       R  K  T  C  M  N  I  M  N  D  P  E  V  K  S  S  H  P  W  F   135
 481  tggcattgaacaagaatacaccctgcttgacattgatgggcacccacttggatggccaaa
       G  I  E  Q  E  Y  T  L  L  D  I  D  G  H  P  L  G  W  P  K   155
 541  gggaggctttccaggaccacaaggaccttactactgtggtgttggtaccaacaaagtgtt
       G  G  F  P  G  P  Q  G  P  Y  Y  C  G  V  G  T  N  K  V  F   175
 601  tggtcgagaagtagtagaagcacattacagagcatgtttgtatgctggtgtgaagattgc
       G  R  E  V  V  E  A  H  Y  R  A  C  L  Y  A  G  V  K  I  A   195
 661  tggtactaatgccgaggttatgcctgctcagtgggaataccaagttggaccatgtgaagg
       G  T  N  A  E  V  M  P  A  Q  W  E  Y  Q  V  G  P  C  E  G   215
 721  cattgagatgggtgatcatctatggatctcaagatatatccttcatcgagttgctgaaga
       I  E  M  G  D  H  L  W  I  S  R  Y  I  L  H  R  V  A  E  D   235
 781  cttccatgttgttgtatcatttgatcctaaacccatgcctggtgactggaatggtgctgg
       F  H  V  V  V  S  F  D  P  K  P  M  P  G  D  W  N  G  A  G   255
 841  tgctcactgcaattacagcaccgttggaatgagagaagaaaatggaatgaggttgatcta
       A  H  C  N  Y  S  T  V  G  M  R  E  E  N  G  M  R  L  I  Y   275
 901  tgaagccattgacaagcttgaaaagaaccatgactaccacatcaagaaatatgaccccaa
       E  A  I  D  K  L  E  K  N  H  D  Y  H  I  K  K  Y  D  P  K   295
 961  gcaaggacaagacaatgcgcgtcggttgactggaaggcatgagacctcaagtatttatca
       Q  G  Q  D  N  A  R  R  L  T  G  R  H  E  T  S  S  I  Y  Q   315
1021  attctctcatggtgtagccaacagaggtgccagtgtaagaatcccacgtcaatgtgctga
       F  S  H  G  V  A  N  R  G  A  S  V  R  I  P  R  Q  C  A  E   335
1081  ggatggcaagggctatcttgaagatagacgaccatcatcaaactgtgatccatacagtgt
       D  G  K  G  Y  L  E  D  R  R  P  S  S  N  C  D  P  Y  S  V   355
1141  tacagaggcaattgtcaggacaactataaagaactacacatgt**tga**taagatggactagt
       T  E  A  I  V  R  T  T  I  K  N  Y  T  C  *                   369
1201  aataattagtagagattattacatataatactgggttcaacatatttatttttaacacctt
1261  caaaaattattgaatgcaatttttaattgacaaggtacctcaacaaatagaatccaacgac
1321  attgtagttttctctttttaatagacatttaatgattctatgagaatcatcatccattga
1381  attcatttaaattcaggggagtactgctgtttctgttgaaattattaatgggaattgcac
1441  gctatttagatttcaaaaatctcacaaagcgtttttttgagattaatgattacaagagttg
1501  tttagttgtatttgatgtttagtctagtactcgtttttatgtgatgtttttgtgacttat
1561  tttgttgtttttagtacttatgtatatagatgactttt**aataaa**gatattttagagatta
1621  aaaaaaaaaaaaaa
```

Figure 2. Nucleotide sequence and deduced amino acids of glutamine synthetase from *Aiptasia pallida* cDNA (Genbank accession number AY472042). The 'atg' and 'tga' specifying the 1107-bp open reading frame (369 amino acids) are indicated in bold. Also bolded is the polyadenylation signal ('aataaa').

untranslated region (UTR) is comprised of 76 nucleotides and includes a context for translational initiation (agagcc**atg**a) that is different than the Kozak (1991) consensus sequence (gcca/gcc**atg**g) observed for vertebrate mRNA. Furthermore, the RLM-RACE protocol is based on the selection of mRNAs with full-length 5′ ends (Schaeffer, 1995); therefore, our results suggest we have completely identified the 5′ UTR, including the transcription start site. In the future, the transcription start site should be verified by conducting a primer extension experiment or a nuclease protection assay (Jones et al., 1994). The 3′ UTR region approximates 450 nucleotides and contains a polyadenylation signal (aataaa) which is 16 nucleotides upstream of the poly(A) tail.

A phylogenetic analysis was conducted using a variety of eukaryotic GS protein sequences from the Genbank database. As presented in Figure 3, this multiple sequence analysis produced a dendrogram that grouped plant and algal GS sequences separate from the GS sequences of *A. pallida* and other organisms. This analysis also showed that *A. pallida* GS clustered into a group that contained fly, sea urchin, mouse, and human sequences as a distinct grouping from yeast and nematode sequences. Based on single comparisons using the GCG program GAP (Womble, 2000) and the organisms listed in Figure 3, the ranking of *A. pallida* GS based on matches of identical amino acids from highest to lowest was: sea urchin (66%), human (65%), mouse (64%), fly (62%), nematode (59%), corn (58%), yeast (56%), alfalfa (56%), red algae (52%), and green algae (51%).

The GS cDNA produced in this study will have a variety of applications to the study of gene expression in the sea anemone, *A. pallida*, including the characterization of RNA by northern-blot analysis and the measurement of RNA levels by means of RT-PCR assays. This cDNA can also be employed as a probe for screening a genomic library for the structural gene and for PCR-based

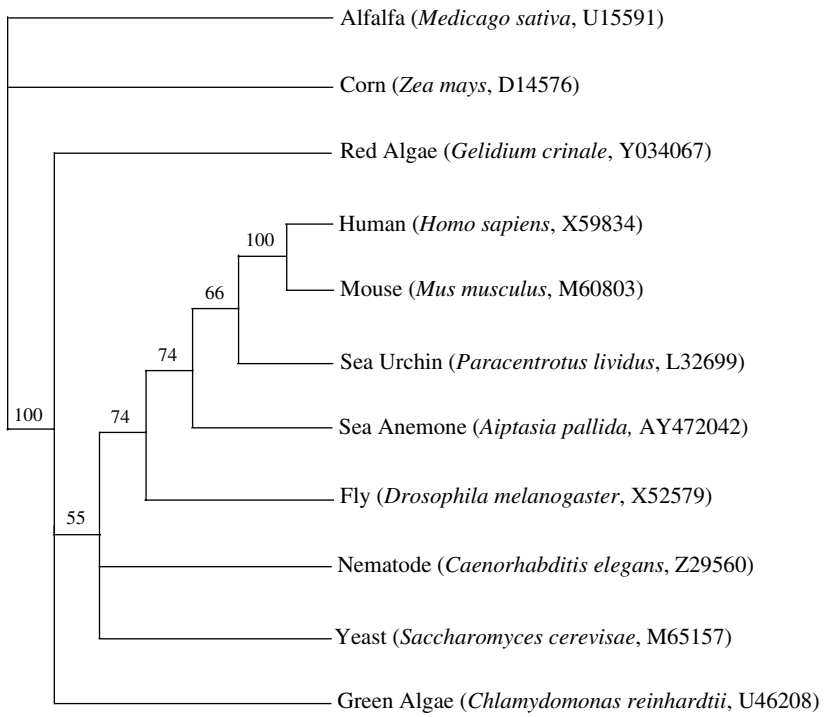


Figure 3. Estimate of phylogenetic relationships among glutamine synthetase protein sequences from 11 organisms. The dendrogram was produced using PAUPDISPLAY and selected sequences from Genbank. The scientific name and Genbank accession number follow each organism (in parentheses). The number at each node of the dendrogram indicates the number of times the partition into the two groups occurred among 100 trees, based on bootstrap resampling.

strategies to identify the promoter sequence for gene expression.

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Part V
Axial Patterning

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The dynamics of multiple mouth formation in *Fungia granulosa*: possible patterning mechanisms

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Key words: morphogenetic gradients, pattern formation, regeneration, scleractinian coral, *Fungia granulosa*

Abstract

We studied patterning mechanisms acting on the formation of new mouths during regeneration of wedge-shaped fragments of the solitary coral *Fungia granulosa*. Mouth formation dynamics was studied in fragments taken from different parts of the adult polyp. The corals were fragmented into central and peripheral fragments in relation to each polyp's centrally positioned mouth. The study revealed that the maintenance of the original body plan and growth axis during the process of regeneration, appears to depend on the existence of the polyp's mouth. When damage to the coral polyp includes only partial damage to the original mouth, the regeneration process is characterized by maintenance of the growth axis and body plan. By contrast, complete removal of the original mouth results in the formation of multiple mouths and is followed by a drastic change in body plan and growth axis. Some of these mouths develop later into new polyps, which eventually detach from the original coral. During the first 2–3 mo of regeneration, 'preliminary' mouths developed and regressed until stabilization was attained. The appearance of new mouths in fragments farther from the original mouth (peripheral fragments) preceded by a week the appearance of new mouths in fragments that were more proximal to the original mouth (central fragments). Additionally, more mouths formed in peripheral than in central fragments. We interpret the results in light of the developmental model suggested by Meinhardt for *Hydra*. The formation of new mouths in regenerating fragments of *Fungia* is explained in terms of a source density gradient field, auto-activation, and lateral inhibition exerted by the original central mouth. These effectors determine the position and timing of mouth(s) development.

Introduction

The role of morphogenetic factors in controlling pattern formation and determining body plan has been widely studied in Hydrozoa. A remarkable volume of research has been devoted to studying the morphogenetic process of development and maintenance of the 'head' region in these organisms (e.g. Wolpert et al., 1974; Sugiyama, 1982; Meinhardt, 1993; Weinziger et al., 1994; Müller, 1995, 1996). Many of these studies have provided the basis for theoretical models of pattern formation, and others identified molecular components involved in relevant processes mainly in *Hydra* and its marine relative *Hydractinia* (e.g. MacWilliams & Fotis, 1974; Shostak, 1974; Wolpert et al., 1974; Sugiyama, 1982; Schummer et al., 1992; Meinhardt, 1993; Shenk et al., 1993; Weinziger et al., 1994; Müller, 1995, 1996; Yan et al., 1995; Grens et al., 1996; Martínez et al., 1997; Mokady et al., 1998; Cartwright et al., 1999).

Since most of the currently available knowledge is based on the body plan of *Hydra*, investigation of cnidarians with different body plans is called

for. Knowledge thus gained may complement our understanding of the role of morphogenetic factors in the establishment and maintenance of body plan in this group.

The discoid scleractinian coral *Fungia granulosa* (Fungiidae) is a solitary, free living coral found in rocky and sandy habitats at a variety of depths in the Indo-Pacific and Pacific Oceans (Highsmith, 1982; Hoeksema, 1988). This is a gonochoric coral that reproduces by releasing gametes to the surrounding water (Kramarsky-Winter & Loya, 1998). The resulting planula embryo settles and metamorphoses into a single polyp, which grows by elongation and forms a calcareous stalk (Fig. 1). At some point the main growth axis changes and the polyp begins growing radially at its upper portion, forming a cap-like thickening (Fig. 1; see Hoeksema, 1988). The cap then detaches from the stalk and becomes free (Fig. 1). Since this coral has a body plan markedly different from hydrozoan polyps, it is interesting as a comparative model for pattern formation.

This coral is especially suitable for studying developmental processes for the following reasons: (1) It is capable of quick regeneration (Chadwick & Loya, 1990); (2) it is easy to manipulate and maintain under laboratory conditions (Kramarsky-Winter & Loya, 1998); and (3) as opposed to most other Scleractinia, it is a large solitary polyp, in which morphogenetic processes are easy to follow.

Though different aspects of regeneration have been studied in Fungiidae (Boschma, 1923; Chadwick & Loya, 1990; Jokiel & Bigger, 1994; Kramarsky-Winter & Loya, 1996, 2000), very little is known about morphogenetic factors and their role in body plan maintenance. As early as 1923 Boschma reported that when the original mouth was functionally eliminated in *Fungia fungites*, buds appeared throughout the polyp surface. Recently, evidence has accumulated further indicating the importance of the mouth tissue in maintaining the body plan in fungiid corals (Jokiel & Bigger, 1994; Kramarsky-Winter & Loya, 1996). Jokiel & Bigger (1994) reported that the formation of new mouths in mouthless fragments of *F. scutaria* occurs along the cut edge proximal to the original mouth. This led them to hypothesize that the presence of the original mouth in fragments prevented formation of additional mouths during regeneration.

The purpose of the present study was to gain further insights into the patterning mechanism behind mouth formation in *Fungia granulosa*. We monitored the development of new mouths in fragments with and without mouth tissues and from different portions of the polyp (peripheral and central fragments) through the first 3 mo of regeneration. The results reveal striking similarities to phenomena observed in head regeneration of *Hydra*.

Materials and methods

Animal collection and maintenance

Individual *Fungia granulosa* (40–50 mm in diameter) were collected from near the H. Steinitz Marine Biological Laboratory (MBL) at Elat, Israel, by SCUBA diving, and brought to the Tel-Aviv University laboratory. Polyps of this size class were chosen because they are characterized by a high rate of regeneration (Chadwick & Loya, 1990). The corals were acclimated in aerated seawater aquaria for 1 week prior to the experiments.

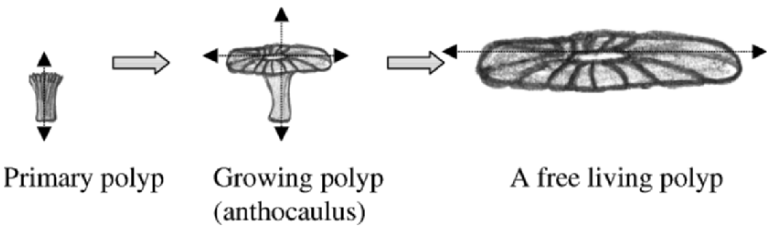

Figure 1. Stages in the development of a young polyp of *Fungia granulosa*. Following settlement and metamorphosis of a planula, a primary polyp (anthocaulus) is formed, with its main growth axis along a stalk. As the apical edge of the stalk broadens, the main growth axis shifts to become radial (from the center of the polyp to its perimeter). This axis is maintained in the detached, free-living polyp. Growth axes are illustrated by dotted arrows.

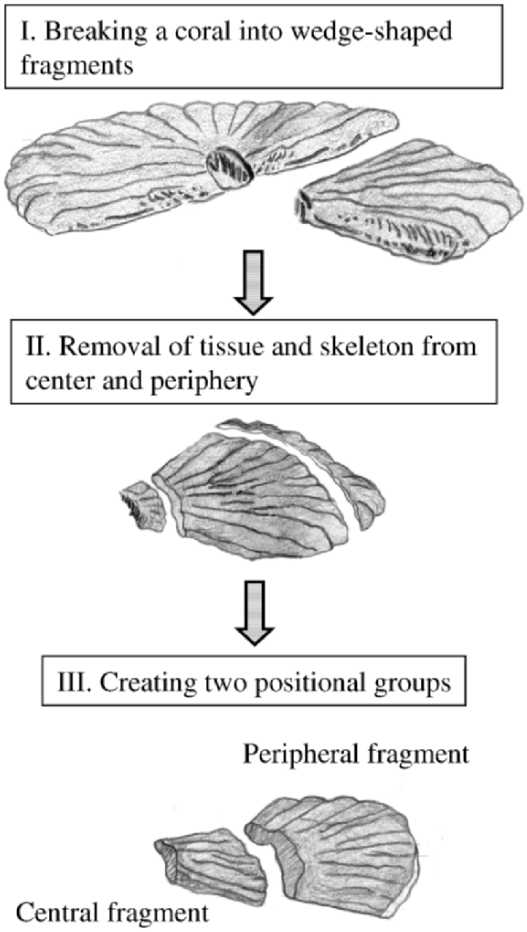
I. Breaking a coral into wedge-shaped fragments
II. Removal of tissue and skeleton from center and periphery
III. Creating two positional groups
Peripheral fragment
Central fragment

Figure 2. Fragment types of *Fungia granulosa* used in this study. A whole coral was broken into a wedge shaped fragment; mouth and peripheral tissues were retained in some of the fragments, while in others they were removed. Mouthless fragments were then broken again into two parts, creating two 'positional' groups of fragments – 'central' and 'peripheral'.

Morphological examination of mouth formation

In order to visualize and follow changes in their tissues, individual corals were vitally stained by immersing them in 1.25 10^{-3} toluidine-blue in 0.45-μm filtered seawater for 24 h. The corals were removed from the staining solution, rinsed several times in clean seawater and maintained in aquaria with clean seawater overnight. The corals were then broken into wedge-shaped fragments using a hammer and a chisel (Fig. 2). In some of the fragments the mouth and peripheral tissues were retained, while in others they were removed (Fig. 2). Mouthless fragments were then broken again into two parts, creating two 'positional' groups of fragments – 'central' (14 fragments) and 'peripheral' (24 fragments) (Fig. 2). Each positional group was subdivided into two size-groups: small (5–10 mm long) and large (10–15 mm long). Each size-group included 6–13 fragments and was kept in a separate aquarium. The dynamics of mouth formation was followed weekly for a period of 3 mo using a dissecting microscope. The results were statistically analyzed (Mann–Whitney *U*-test) using 'Statistica' (version 3) software.

Results

Regeneration of fragments with mouth tissues

The process of tissue regeneration in this coral entailed an initial sloughing off of torn tissue and cellular debris from the area of damage, followed by coverage of the bared skeleton by the coral tissue. Once the tissue covered the bare skeleton, regeneration of mouth tissues began. Following mouth tissue repair, new septae developed around the repaired area (Fig. 3A–C). The symmetry of the newly formed septa was identical to that of the old septa resulting in a conservation of the fragment's growth axis (Fig. 3C).

Regeneration of mouthless fragments

In mouthless fragments, once the tissue fully covered the bare skeleton, new mouths began to form. This occurred at the facet most proximal to the original mouth (Fig. 3D–F). We distinguished developing mouth tissue from tissue tears by the development of thick, densely stained tissue, at the perimeter of a distinct aperture. Once the new mouths were distinguishable, new tentacles and septae started to develop. The plane of growth of the new septae was perpendicular to the direction of growth of the original septae, reflecting a drastic change in the growth axis of the regenerating fragments (Fig. 3F).

The development of the new mouths was a dynamic process. 'Preliminary' mouths appeared and disappeared for a period of 2–3 mo until stabilization. Eventually one or more mouths took precedence over the rest (Fig. 4). The appearance of new mouths in fragments farther from the

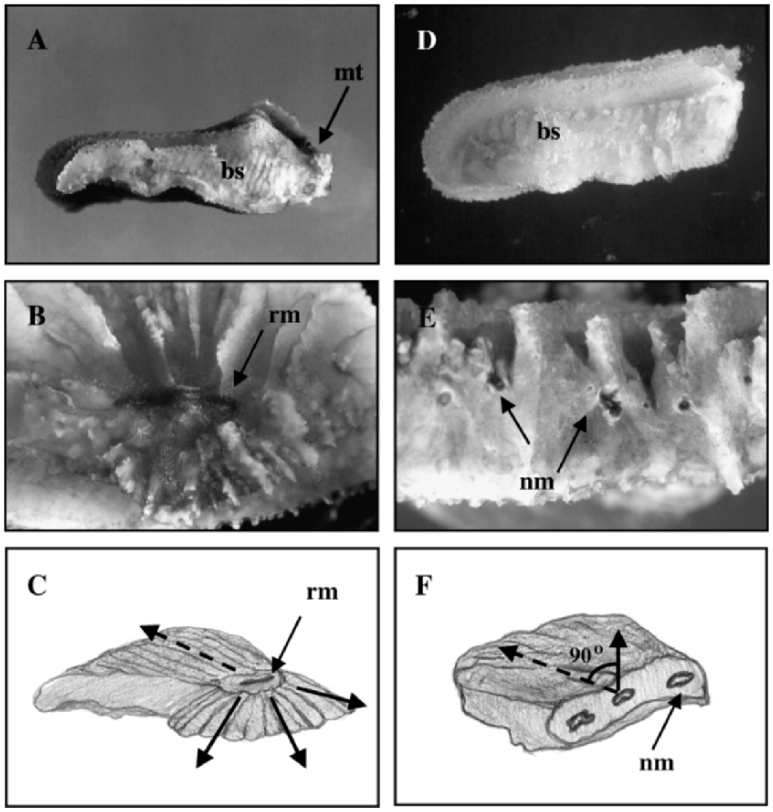

Figure 3. Mouth formation in regenerating fragments of the coral *Fungia granulosa*. (A) Side view of a wedge shape fragment retaining some mouth tissue following breakage. (B) Photo of the wedge following regeneration; note the new septae in the fragment regenerating in the presence of remnants of the original mouth. (C) Illustration of the repaired mouth in a wedge shaped fragment. (D) Side view of a mouthless fragment following breakage. (E) Photo of the front facet of the wedge following tissue regeneration. Note muiltiple new mouths. (F) Illustration of multiple-mouths developing on the front facet of a mouthless fragment. Old and new growth axes are represented by dashed and solid arrows, respectively (C, F) mt, mouth tissue; rm, regenerated mouth; bs, bare skeleton; nm, new mouth.

original mouth (peripheral fragments) preceded by a week the appearance of new mouths in fragments that were more proximal to the original mouth (central fragments) (Fig. 4A and B).

The highest number of mouths observed in small and large central-fragments were 1.6 ± 0.47 and 2.5 ± 0.66 respectively. The highest number of mouths observed in small and large peripheral fragments were 2.1 ± 0.74 and 3.55 ± 1.16 respectively. As can be seen in Fig. 4 these peaks were evident at different time periods during the process.

In central fragments this difference was significant 35 d after the breakage (Mann–Whitney *U*-test $0.01 < p < 0.05$) but not significant 74 and 96 d after the breakage (Fig. 4A). In peripheral fragments this difference was significant 62 and 72 d after the breakage (Mann–Whitney *U*-test $0.01 < p < 0.05$) but not significant 96 d after the breakage due to stabilization (Fig. 4B). These time periods characterize the two stages of the process of new mouth development in regenerating coral fragments. The first part of the process is termed the 'dynamic stage', in which mouths appear and, disappear. The second part is termed the 'stabilization stage', in which there is stabilization in the number of mouths (Fig. 4).

Discussion

Most of the extensive literature documenting the regeneration process in scleractinian corals focuses

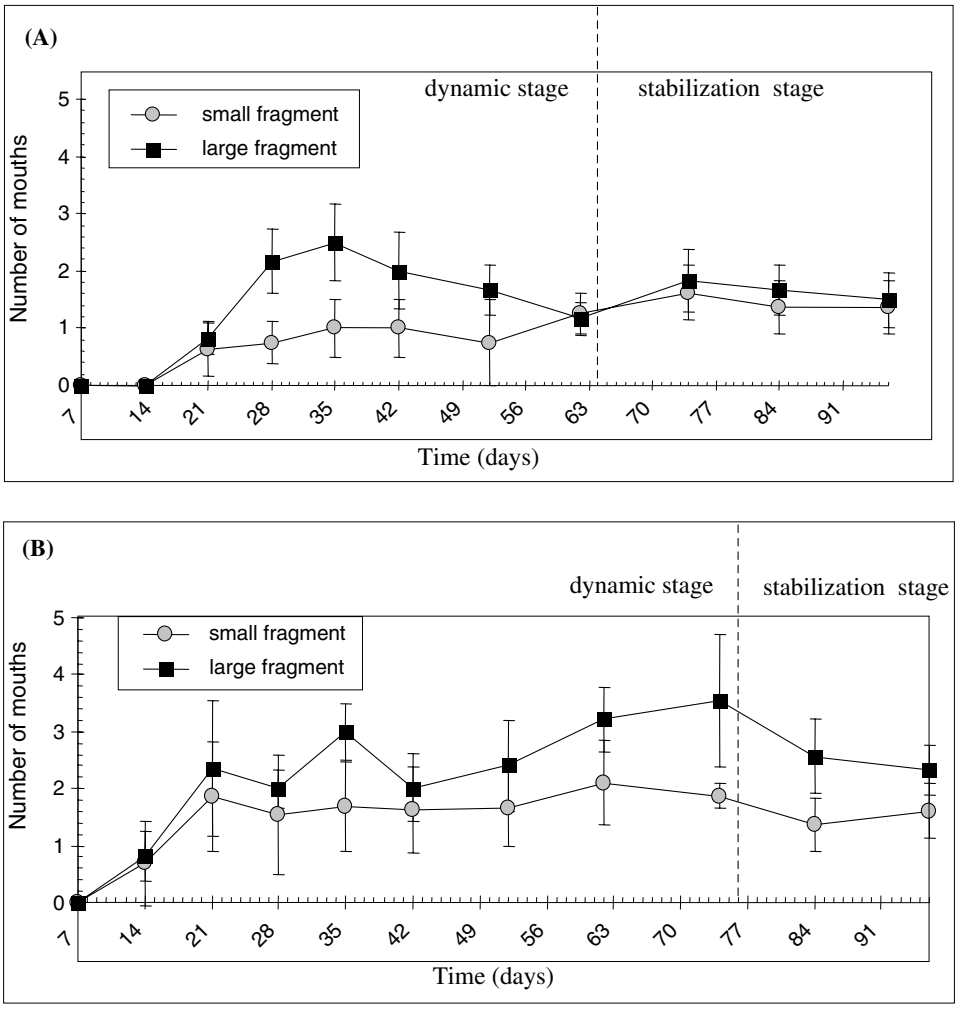

Figure 4. Development of new mouths in fragments of the coral *Fungia granulosa*. The dynamics of new mouth development in different fragments (average ± SD), occurring during the first 3 mo of regeneration. (A) Central fragments (small and large). (B) Peripheral fragments (small and large).

on physiological and environmental aspects of the process (Highsmith, 1982; Jokiel & Bigger, 1994; Kramarsky-Winter & Loya, 2000). Very little is known about the cellular level or the patterning mechanisms behind this process.

Previous studies showed the importance of the presence of mouth tissues in inhibiting the development of new mouths (and buds) in many fungiid species (Boschma, 1923; Chadwick & Loya, 1990; Jokiel & Bigger, 1994; Kramarsky-Winter & Loya, 1996). The present study examined the dynamics of this phenomenon in regenerating fragments of the coral *Fungia granulosa*, in order to shed light on patterning mechanisms underlying it. We interpret our observations in light of the model suggested by Meinhardt (1993), involving a source-density gradient. Analogous to morphogenetic gradients described for *Hydra* (see Wolpert et al., 1974; Meinhardt, 1993; Broun & Bode, 2002), we propose that in *Fungia*, morphogenetic effects are exerted by the central mouth along the coral's main growth axis which, unlike in *Hydra*, is radial (see Fig. 1).

In *Fungia* the morphogenetic effect of the mouth seems to act similarly to that of the 'head activator' in *Hydra* (Meinhardt, 1993). Like the head-activator, the mouth effect produces both auto-activation and lateral inhibition, inhibiting

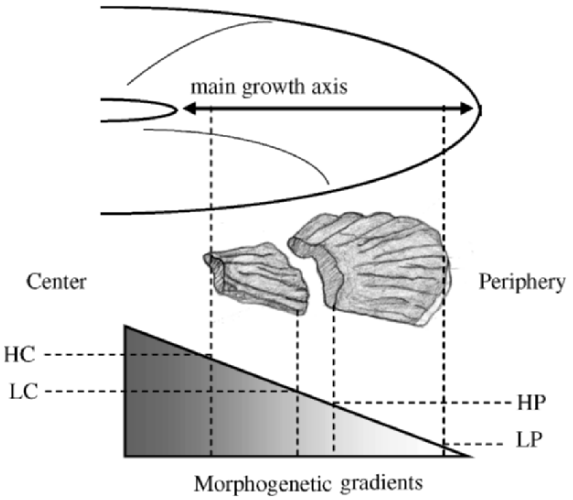

Figure 5. Morphogenetic gradients along the growth axis of *Fungia granulosa*. The gradients of source density and mouth inhibitory effect originate in the mouth tissue and their levels decrease gradually toward the periphery. The levels expected at different locations in fragments are coded as follows: HC, LC – highest and lowest level in a central fragment, respectively; HP, LP – highest and lowest level in a peripheral fragment, respectively.

the formation of additional mouths in its vicinity (Meinhardt, 1993). Thus fungiid fragments that retain remnants of mouth tissue will reform a single mouth because this tissue produces enough of an effect to activate mouth repair while concurrently inhibiting the formation of new mouths in its vicinity. However, complete removal of mouth tissue from the coral fragment will cause the removal of the source of the mouth inhibitory effect, allowing formation of new mouths. The present study shows that removal of mouth tissue together with peripheral tissue from a fragment still results in new mouths developing in positions most proximal to the original mouth. This may indicate that the 'positional value' of the tissue (see Wolpert et al., 1974; Wolpert, 1998; Meinhardt, 1993) is preserved.

In both central and peripheral fragments mouths formed only at the facet most proximal to the original mouth. Like in the model presented by Meinhardt (1993) for head activation in *Hydra*, *Fungia* mouth tissue may be responsible for establishing a source-density gradient, involved in regulating the effects of mouth activation and inhibition. Mouth(s) will form only at the highest relative source density. As shown by Fig. 5, regardless of fragment position (central versus peripheral), highest relative source density is expected at the facet most proximal to the original mouth.

The appearance of new mouths in peripheral fragments preceded the appearance of new mouths in central fragments (Fig. 4). This observation may be explained by a lower level of inhibitory effect exerted by the original mouth tissue on the development of new mouths (lateral inhibition), associated with the highest source density in each fragment (Fig. 5). The inhibitory effect decreases radially with distance from the original mouth. Thus, in peripheral fragments, highest source density is lower than that in the central fragments. Not only the timing of development of new mouths, but also the higher number of mouths, in peripheral fragments (Fig. 4) may reflect a lower level of inhibition (Fig. 5).

The dynamics of the process by which new mouths develop and regress may imply local differences in morphogenetic effectors but may also be the result of stochastic processes. New mouths thus formed start exerting an effect on their surroundings, inducing the lateral inhibition of mouth formation. This eventually causes the observed 'stabilization' of the number of mouths, when one or more of the newly formed mouths become dominant (Fig. 4).

The drastic change in the polyp fragment's growth axis can also be explained following the rationale detailed above. Histological examination of the process of regeneration reveals tissue stretching down and up the bare coral skeleton toward the midline of the broken facets (Shimony, 1999). Thus, the cells featuring highest source density reach this midline prior to the formation of new mouths (Fig. 3). The position of the new mouths dictates the positions and postures of newly formed septae and tentacles, resulting in the observed right-angle shift in growth axis. Thus, the mouth region of *Fungia* seems to act as an organizer, similar to the hypostome in *Hydra* (see Broun & Bode, 2002). The new growth axis is similar to the one observed in budding *Hydra*, as the buds formed are perpendicular to the growth axis of the parent body column.

Clearly, the current research is but a first step towards understanding the forces that drive pat-

tern formation in scleractinian corals. Experiments comparable to the exhaustive ones carried out in *Hydra*, including grafting and examination of the molecular mechanisms underlying pattern formation, are necessary in order to assess just how similar the processes of morphogenesis are between *Hydra* and *Fungia*.

Acknowledgments

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Evolution and dynamics of branching colonial form in marine modular cnidarians: gorgonian octocorals

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Key words: colonial organisms, heterochrony, Cnidaria

Abstract

Multi-branched arborescent networks are common patterns for many sessile marine modular organisms but no clear understanding of their development is yet available. This paper reviews new findings in the theoretical and comparative biology of branching modular organisms (e.g. Octocorallia: Cnidaria) and new hypotheses on the evolution of form are discussed. A particular characteristic of branching Caribbean gorgonian octocorals is a morphologic integration at two levels of colonial organization based on whether the traits are at the module or colony level. This revealed an emergent level of integration and modularity produced by the branching process itself and not entirely by the module replication. In essence, not just a few changes at the module level could generate changes in colony architecture, suggesting uncoupled developmental patterning for the polyp and branch level traits. Therefore, the evolution of colony form in octocorals seems to be related to the changes affecting the process of branching. Branching in these organisms is sub-apical, coming from mother branches, and the highly self-organized form is the product of a dynamic process maintaining a constant ratio between mother and daughter branches. Colony growth preserves shape but is a logistic growth-like event due to branch interference and/or allometry. The qualitative branching patterns in octocorals (e.g. sea feathers, fans, sausages, and candelabra) occurred multiple times when compared with recent molecular phylogenies, suggesting independence of common ancestry to achieve these forms. A number of species with different colony forms, particularly alternate species (e.g. sea candelabrum), shared the same value for an important branching parameter (the ratio of mother to total branches). According to the way gorgonians branch and achieve form, it is hypothesized that the diversity of alternate species sharing the same narrow variance in that critical parameter for growth might be the product of canalization (or a developmental constraint), where uniform change in growth rates and maximum colony size might explain colony differences among species. If the parameter preserving shape in the colonies is fixed but colonies differ in their growth rates and maximum sizes, heterochrony could be responsible for the evolution among some gorgonian corals with alternate branching.

Introduction

Branching patterns, or tree-like forms, are everywhere in nature: river basins, organ vessels, trees, fungi, algae, hydroids, and corals, just to mention some. Most organisms on earth contain branching networks. Still, regardless of the recurrence of this pattern in multiple species and levels of organization, we do not clearly know how this pattern forms or evolves (see reviews in Buss, 2001; Lasker & Sánchez, 2002; Rinkevich, 2002). This is of great significance for the understanding of marine sessile modular organisms such as algae, sponges, hydroids, bryozoans, and corals because growth and

form are their main means of interaction with the environment (e.g. Jackson, 1977; Hughes, 1983). Although modular organisms seem physiologically simple and repetitive, development and branching in these systems is evidently complex and dynamic (e.g. Kaandorp & Kübler, 2001).

The greatest difficulty in understanding the growth and form of colonial organisms is the absence of an obvious causal mechanism producing form, which overall is a general problem in evolutionary developmental biology. In-depth developmental studies have unavoidably revealed mechanisms concerning cell division, migration, and differentiation. Certainly, many genes play an important role in pattern differentiation, and for individual organisms (or individual modules). Homeobox genes, for example, play an important role in pattern formation and cell fate during development (see reviews in Popadic et al., 1998; Purugganan, 1999). It is important to note that these genes can act only after several other genes and maternal factors are expressed. Therefore, the process is complex enough to not be controlled by single molecules. Nonetheless, both individual and colonial forms of cnidarians exhibit most Hox/Parahox gene families present in higher metazoans (e.g. Finnerty & Martindale, 1997; Kuhn et al., 1999). These genes have been used as 'bottom-up' exemplars to understand the development of the individual polyps as well as during the polyp-medusa metamorphosis in hydroids (e.g. Aerne et al., 1996; Cartwright et al., 1999) and are promising for understanding the expansion of connecting colonial networks (e.g. Cartwright & Buss, 1999). Nonetheless, branching patterns seem to be an emergent property of modular replication in colonial organisms, which is not entirely understood. This paper reviews and summarizes recent results in the theoretical and comparative biology of modular organisms, which are 'top–down' approaches to understanding the problem of patterning and development of modular colonial organisms.

Modular and colonial traits in octocorals

Colonial organization in gorgonian octocorals presents high complexity at several levels. Octocoral polyps (modules) always have eight pinnate tentacles, and they are monomorphic, each capable of all physiological functions, i.e. reproduction, feeding, excretion, defense, etc. (e.g. solitary octocorals: Bayer & Muzik, 1976). The polyps are immersed in coenenchyme, a mesoglear layer mix of calcareous sclerites and the non-cellular portion of the middle layer. The distribution of polyps on the branches varies between species, ranging from bilateral series of polyps along the edges of compressed branches to a random or uniform distribution over a cylindrical branch. Nonetheless, the highest complexity is observed in the process of branching; over 20 qualitative branching patterns have been identified by taxonomists (Bayer et al., 1983). The polyps are connected to each other by a network of stem canals (Bayer, 1973). The connections among polyps continue along the branch axis and form a branching tree-like structure (Sánchez & Lasker, 2004).

In the study of form evolution, it is important to know if characters producing the form are independent of each other or evolve as integrated sets. In modular colonial organisms, which form by the repetition of identical units, the emergent forms are usually multi-branched complex networks. To examine the patterns of integration among morphologic traits in Caribbean octocorals, Sánchez & Lasker (2003) compared five morphologic traits across 21 species, correcting for the effects of phylogenetic relationships using molecular phylogenies (Sánchez et al., 2003b). Although all characters exhibit associations with each other, a multivariate analysis, correcting for conditional independence (e.g. partial correlation coefficients), showed the strongest integration among the colony level (network) characters. This is a quantitative and comparative demonstration that branching characters within colonial organisms are independent of characters of the modules, which has also been observed in experimental studies of hydroid colony growth (e.g. Müller et al., 1987; Kossevitch et al., 2001).

Other marine branching invertebrates such as colonial bryozoans present such integration between the different branching angles that their correlation generates a morphospace of realistic colonies (McKinney & Raup, 1982). In trees, interspecific comparisons among multiple traits such as twig, leaf, seed, and branches have exhibited correlated evolution (Ackerly & Donoghue, 1998). Plasticity in plant populations also shows

morphologic integration (e.g. Waitt & Levin, 1993; Pigliucci & Marlow, 2001). The co-evolution of modules of characters within the structure appears to be a common feature of species exhibiting multi-branched tree-like networks.

Colonial growth in gorgonian octocorals: branching

The usage of ordering schemes, assuming growth of the network through bifurcation, does not realistically explain the branching and/or growth of modular marine organisms such as Caribbean octocorals (Sánchez et al., 2003c). Branching in gorgonian octocorals is a subapical process where all the branches are structurally the same but some branches, 'mother branches', produce new branches, 'daughter branches', at roughly fixed distances or internodes (Fig. 1). Branching does not follow a bifurcation-based process but a dynamic production of daughter branches from established mother branches. According to Sánchez (2002) and Sánchez et al. (2004), branching starts at a constant rate, r (daughter branches year^{-1}), at the first primordial mother branch. As the colony grows, a few daughter branches turn into mother branches (Fig. 1) and start branching at a rate that maintains a fixed ratio between the total number of branches and the number of mother branches, c. This parameter is critical for the process of branching since octocoral colonies grow in a self-organized dynamic process maintaining as constant as possible the ratio of mother to daughter branches. Colony growth proceeds in this manner, but slows asymptotically as the colony reaches a maximum number of mother branches, k, which seems to be related to branch interference and allometry (Sánchez et al., 2004). Branching can be modeled with the simple parameters r, c, and k with this recurrence expression (1):

$$S_{t+1} = S_t + \frac{S_t r}{c}\left(1 - \frac{S_t}{k}\right), \tag{1}$$

where S is the number of mother branches. This expression predicts sigmoid- or logistic-like accumulation of branches through time, which meets the predictions from empirical observations from multiple growth studies found in the hydroid and octocoral literature (e.g. Kauffmann, 1981; Stebbing, 1981; Coma et al., 1998). This approach to branching explains how modular colonial organisms preserve form during development,

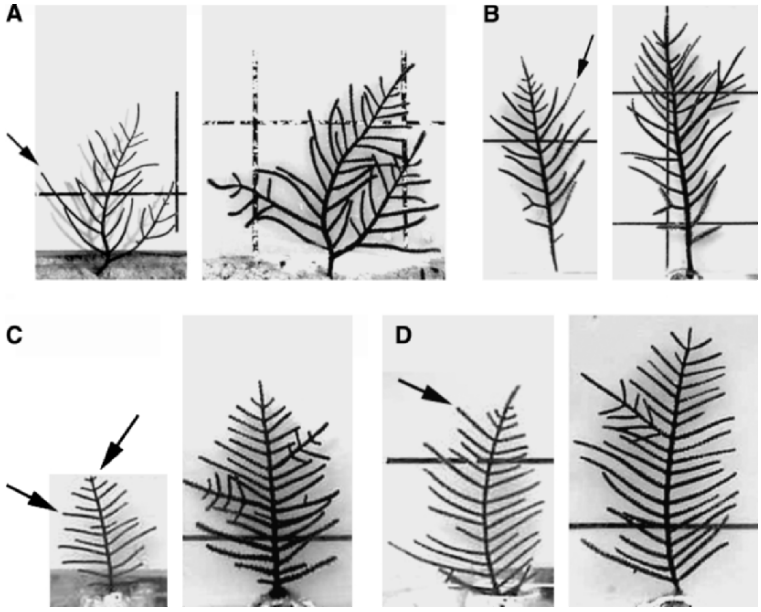

Figure 1. Sequential images (Dec-1999 and Dec-2000) of several Caribbean gorgonian octocorals (Riding rock reef, 15 m, San Salvador, Bahamas). (A) *Pseudopterogorgia americana*. (B) *P. elisabethae*. (C and D) *P. bipinnata*. Arrows show the daughter branches that developed into mother branches after a 6 month period.

independently of the maximum size allowed by ecological/physiological constraints. This understanding of branching suggests a new perspective on the functional and evolutionary biology of these colonial organisms.

Evolution of branching in gorgonian octocorals

The recent molecular phylogenies of octocorals show how branching could evolve independently multiple times from non-branching hypothetical ancestors. Analyses of ribosomal DNA sequences, from either mitochondrial lsu-rRNA (16S) or nuclear ssu-rRNA (18S), have revealed similar phylogenetic relationships supporting two main groups of branching gorgonian corals: suborders Calcaxonia and Alcyoniina-Holaxonia (Sánchez et al., 2003a). This molecular evidence corroborated results uncovering the evolution of two independent and convergent groups of branching octocorals, which have analogous rigid axial structures. These octocoral clades were also found in both France et al. (1996) and Berntson et al. (2001) with low statistical support. In addition, the two clades have qualitative differences in their predicted RNA secondary structures (Sánchez et al., 2003a). Likewise, comparing major morphological characters revealed that morphological complexity in both axial structures and sclerites developed in parallel in both Calcaxonia and Alcyoniina-Holaxonia clades. Convergence of branching patterns among octocorals has also been observed in closely related species.

Modular colonies of Caribbean gorgonian corals are organized into unconventional branching networks (e.g. Fig. 1). There are three basic branching patterns among gorgonians: sea fans (reticulate), sea feathers (pinnate), and alternate (sea candelabra/sausages). These branching patterns occurred multiple times in a gorgonian molecular phylogeny (NADH dehydrogenase subunits 2 and 6 [*ND2, ND6*] and mutS homolog [*msh1*]: Sánchez et al., 2003b), suggesting that these forms can arise independently of common ancestry (Fig. 2A). Reticulate species could have both pinnate and reticulate relatives, whereas pinnate forms only alternate ancestors (Fig. 2A). Changes in colonial form among modular organisms can reflect genetic differences, the product of plastic developmental responses to particular environments, and there is the possibility of genotype-by-environment interactions as well. All paths can lead to the association of particular branching strategies with specific environments. Similarly, forms such as the reticulate fans found across octocoral genera are the result of convergent evolution. Bayer (1953) first proposed convergent evolution for gorgonian colony architectures such as sea fans (*Pacifigorgia* spp. and *Gorgonia* spp.) and sea leaves (*Phycogorgia* spp. and *Phyllogorgia* spp.). Bayer's hypothesis is corroborated by a gorgonian molecular phylogeny (Sánchez et al., 2003b). The differences within each particular branching pattern, on the other hand, can be explained as differences in the branching dynamics.

The ratio between total and mother branches or *c*, an indicator of branching form and critical value for branching, presents an interesting correspondence according to the branching pattern. Caribbean gorgonian corals with alternate or reticulate branching exhibited low values of *c* ranging from 2 to 5 (Fig. 2C). The six species with pinnate branching all had *c* values higher than 13. Mapping these two characters on the phylogenetic hypothesis, it was clear that pinnate branching is associated with high *c* values (Fig. 2A). This association appears to be independent of phylogeny since *Muriceopsis flavida* and *Pseudopterogorgia* spp. both arose from different, presumably alternate, ancestors. A number of reticulate sea fan species could have pinnate ancestor. The one exception to the pattern was the reticulate sea fans *Pacifigorgia elegans* and *P. stenobrochis*. *Pacifigorgia* spp. had a sister relationship (*Leptogorgia virgulata*) with alternate branches, and traces of alternate branching are still visible in *Pacifigorgia* spp. branching patterns (Fig. 2A). *Gorgonia* spp. comprise analogous reticulate sea fans with both clear pinnate branching and pinnate ancestors, which is evident in their sister relationship with some pinnate gorgonians (*Pseudopterogorgia* spp.) and traces of pinnate branching in their networks (Fig. 2A and B).

Pinnate gorgonians were associated with changes in the branching parameter *c*, (the ratio of total branch to mother branch). Therefore, the change from alternate to pinnate could involve a duplication in the set point of branching that changed completely the branching function and growth/form relationship. Forms with alternate

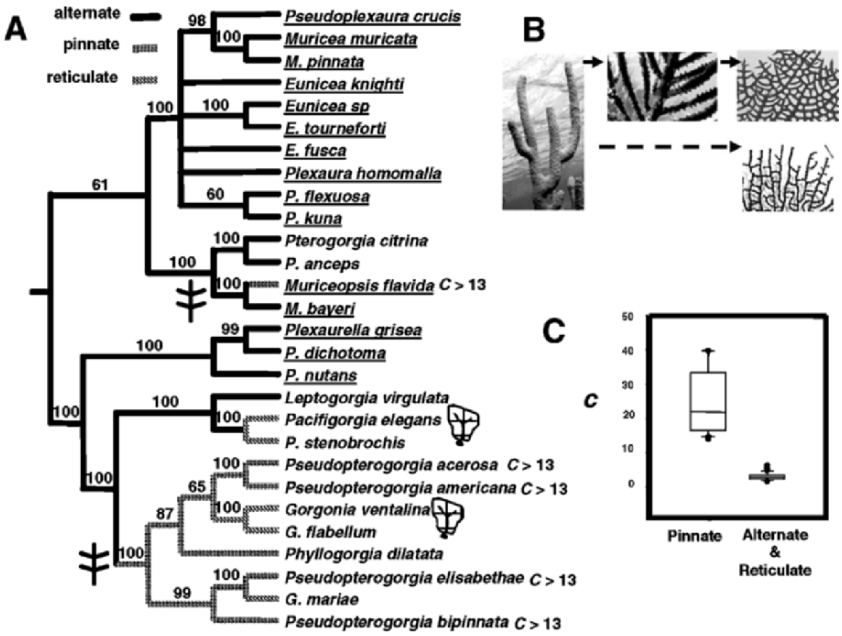

Figure 2. (A) Bayesian-estimated Likelihood tree (HKY) using nucleotide partition by positions (NADH-dehydrogenase subunits 2 and 6, and mutS homolog [msh1], 1633 bp); above node support is the 50%-majority rule consensus from 10,001 sampled trees generated by PAUP* (see details in Sánchez et al., 2003b). Branches are showing a parsimonious distribution of the character type of branching. The pinnate or fan sketches indicate the multiple origins of sea feathers and fans respectively. Underlined species names correspond to nominal plexaurid octocorals, otherwise they are Gorgoniidae. (B) Hypothetical trajectories among different branching types accordingly. Left, *Plexaura flexuosa*-alternate; middle, *Pseudopterogorgia americana*-pinnate; upper right, *Gorgonia ventalina*-reticulate-pinnate; lower right, *Pacifigorgia elegans*-reticulate-alternate. (C) Box plots from the distribution and variance of *c* values among the 24 studied species. The median line is inside the 25th and 75th percentiles with external error bars at the 10th and 90th percentiles.

branching always exhibited low values and variances in *c,* whereas pinnate forms had high values of *c* (Fig. 2B). *C* is an indicator of the form or the relationship between form and growth. If different colonies have the same *c* values, the topological relationships among branches will be nearly identical, differing only in the branching (=growth) rate and the maximum colony and module sizes. This seems to occur among gorgonians with alternate branching. Among these species some colony forms are augmented versions of a small segment of a few branches of small highly branched species (e.g. Fig. 3). The notable conservation in this trait suggests some canalization (see review in Debat & David, 2001) in many species by maintaining the same developmental outcome despite the change in the traits involved. Alternatively, the presence of physical developmental constraints (see review in Resnik, 1995) acting on branching properties could keep certain parameters constant across species. In contrast, pinnate branching species exhibit a wide range of *c* values, suggesting an absence of constraining and canalizing factors from one end or just a difference in relative variability because they have more branches (e.g. Lewontin, 1966). Due to the low number of sea fans included, it was not possible to see if there is a link between fixed *c* values across species and the reticulate form.

Differences in colony size and the similarities among alternate species could be the product of heterochrony. As in individual organisms (e.g. Gould, 1977; McNamara, 1995), the timing and rate of colony development in marine invertebrates can be studied in the context of heterochrony (Pandolfi, 1988). Alteration of the timing of different aspects of colony development may result in adult organisms that are similar to juveniles of other species or vice versa (McKinney, 1988). In addition, the germ line may mature early or late during development as another outcome of evolution through heterochrony. Changes in colony

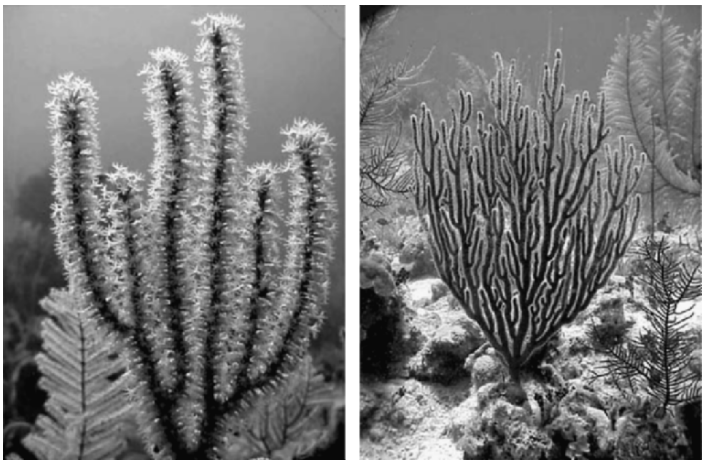

Figure 3. Adult colonies of alternate branched sea candelabrum octocorals from the Caribbean Sea. Left, colony of *Eunicea laxispica*, 30 cm height, Carrie Bow Cay, Belize; Right, colony of *Plexaura flexuosa*, 120 cm height, San Salvador, Bahamas.

architecture have been experimentally induced by accelerating and decelerating growth rates of colonies from the same species (Blackstone & Buss, 1993; Blackstone, 1999, 2000). Those experimental findings demonstrate that uniform change in the rate of colony development generates different colony forms though heterochrony. However, before we can suggest that heterochrony has driven the process leading to the evolution of colony form among alternate gorgonians, we must demonstrate that the developmental trajectories follow the same growth function (Rice, 1997). Interestingly, growth of gorgonian corals with similar values of c (e.g. $c \sim 2.9$) would branch in a simpler manner (2):

$$S_{t+1} = S_t + \frac{S_t r}{2.9}\left(1 - \frac{S_t}{k}\right). \quad (2)$$

Different branching species that had similar values of c would, therefore, vary as a function of their growth rates or maximum sizes, which are examples of heterochrony. Additionally, maximum size as well as the size of first reproduction in gorgonian corals seems to be very predictable and species-specific (e.g. Kalpela & Lasker, 1999). Variation in these traits could provide additional sources of heterochrony (e.g. progenesis). For instance, Figure 4 shows a hypothetical heterochrony example for the species of Figure 3, which both have an empirical value of $c = 2.9$, assuming equal branching rates, r, for the two species but different k values. The latter values seem to be affected and correlated to other parameters from

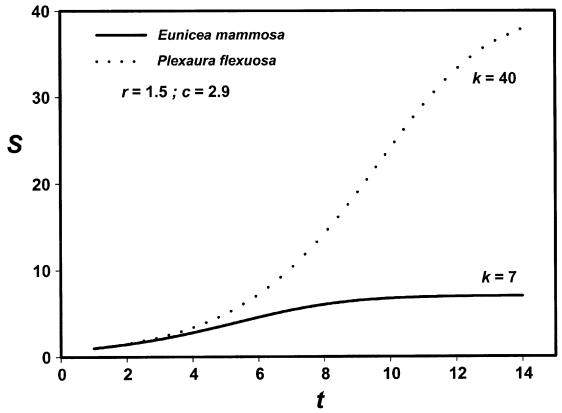

Figure 4. Results from the iteration (14 times) of the model (2) using different values of k corresponding to the species from Figure 3.

the modular construction of the colony (e.g. branch/polyp/internode size, axial material resistance, etc.), which are different between the two species. Further study on the maximum and reproductive colony/module sizes as well as on branching rates could provide an assessment on the occurrence of heterochrony for the evolution of colony form in modular organisms.

Branching in modular organisms: what is particular?

A feature that is characteristic of modular colonial organisms is the presence of supra-modular levels of organization creating new modular structures.

This emergent level of integration is produced by the branching process itself and is not a necessary outcome of the module replication process (Sánchez & Lasker, 2003b). The occurrence of these mostly independent levels of integration in branching Caribbean octocorals presents the question if variation in these traits, either within or among species, can be related to environmental variation operating *via* selection and/or morphologic plasticity. The different levels of modularity found across species have also been noted at the intraspecific level (Lasker & Sánchez, 2002). There is a partitioning in the levels of growth organization, in individual branches, and in the colony as a whole (Lasker et al., 2003). Therefore, not just a few changes at the module level could generate changes in colony architecture, suggesting an uncoupled developmental patterning for the polyp and branch level traits.

Colony growth and form in gorgonian octocorals can be predicted by the dynamic interaction of a few parameters. The changes in these parameters show correspondence with changes in the branching pattern and colony form. The dynamic interaction of some of the parameters suggests a great deal of heterochrony, explaining the evolution of forms that maintain the same branching hierarchy (e.g. ratio of mother to total branches). These parameters can be considered as intrinsic characterizations of the processes that control branching, and this intrinsic aspect of branching can be regarded as genetic in nature (Rinkevich, 2002). Nonetheless, extrinsic effects also have an important effect on colony form but should be constant for a given colony as part of its surrounding environment.

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The role of polyp-stolon junctions in the redox signaling of colonial hydroids

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Key words: epitheliomuscular cell, mitochondria, *Podocoryna*, *Podocoryne*, reactive oxygen species

Abstract

An encrusting colonial hydroid can be regarded as a network of polyps or 'mouths' connected by tube-like stolons. The success of the colony crucially depends on putting these mouths where the available food is. Feeding-related perturbations may provide important signals in this regard. After feeding, polyps contract regularly, dispersing food throughout the colony via the gastrovascular fluid. Mitochondrion-rich epitheliomuscular cells concentrated near polyp-stolon junctions likely drive these contractions. Putatively, the redox state of these cells may influence colony-level form. For instance, the metabolic demand associated with feeding-related contractions results in mitochondria that have relatively oxidized electron carriers and produce lesser amounts of reactive oxygen species (ROS). ROS or other redox-sensitive molecules emitted from polyp-stolon junctions into the gastrovascular fluid may provide stolons with signals influencing elongation, branching, and regression. Treatments of colonies with anti-oxidants cause peripheral stolon tips to rapidly regress. This regression appears to be an active process involving a flux of locally produced peroxides and cell and tissue death. At the same time, polyps and stolon tips in the center of treated colonies remain healthy. 'Sheet-like' growth of short, branched stolons ensues. Signals that inhibit the outward growth of stolons may lead by default to the concentrated growth of stolons and polyps in food-rich areas. ROS may mediate signaling mechanisms involving nitric oxide, programmed cell death, a variety of redox-regulated proteins, or all of these.

Introduction

For a heterotrophic organism, a mouth is a major innovation. A mouth allows feeding on relatively large prey items and the sequestration of large amounts of energy. Nevertheless, to be useful, mouths must be effectively deployed. If the supply of prey items is temporally or spatially variable, this problem is especially acute, particularly for a sessile organism such as a colonial hydroid that relies on prey items for reduced carbon substrate. In such organisms, signaling provided by the food itself may greatly simplify the difficulties of mouth placement. While there are a number of potential mechanisms by which food-related signaling could be achieved (Blackstone, 2001), the polyp contractions that begin shortly after feeding (Dudgeon et al., 1999) will be the focus here. These contractions seem to be driven in large part by mitochondrion-rich epitheliomuscular cells (EMCs) concentrated near polyp-stolon junctions (Blackstone et al., 2004). The metabolic demand associated with feeding-related contractions leaves these mitochondria relatively oxidized and diminishes the formation of reactive oxygen species (ROS; Blackstone, 2001, 2003). Here hypotheses will be developed for potential mechanisms linking redox state and ROS to colony-level form.

If the mitochondrion-rich EMCs at polyp-stolon junctions are involved in colony-wide patterning, signals must be transmitted from these junctions to actively growing stolons, for it is the

growth of these stolons that will determine colony form. If peripheral stolons elongate without branching, 'runner-like' growth ensues. If peripheral stolons regress or branch, or both, 'sheet-like' growth ensues. Whether from mitochondria or other sources, ROS can participate in a broad range of signaling pathways, including interacting with reactive nitrogen species (RNS; Levonen et al., 2001), serving as a trigger for programmed cell death (Duan et al., 1999; Ueda et al., 2002), and oxidizing the amino acid cysteine to change the conformation of the active sites of proteins (Georgiou & Masip, 2003; Salmeen et al., 2003; van Montfort et al., 2003; Woo et al., 2003; Wood et al., 2003). ROS are particularly useful in signaling cascades because they are, by definition, reactive and thus quickly dissipate both temporally and spatially. Here, several common signaling pathways will be briefly reviewed, and experiments with anti-oxidant treatments of the colonial hydroid *Podocoryna* (= *Podocoryne*) *carnea* Sars will be presented (compare to Jantzen et al., 1998). Mechanisms by which polyp-stolon junctions may mediate growth and patterning in hydroid colonies are then discussed.

ROS and signaling pathways

ROS and RNS

It is currently widely accepted that nitric oxide (NO) acts as an inter- and intracellular messenger. Originally identified as a vasodilator, NO also interacts extensively with mitochondria, e.g. at high levels inhibiting cytochrome oxidase, while at low levels up-regulating PGC-1α, a transcriptional coactivator that regulates mitochondrial biogenesis (e.g. Brown, 2003). In turn, ROS produced by mitochondria can interact with nitric oxide and other RNS in a variety of ways (Levonen et al., 2001). A complex web of small-molecule signaling emerges.

ROS and programmed cell death

In animal cells, mitochondria integrate various internal signals and, in response, may release factors such as cytochrome c that activate caspases and initiate programmed cell death. Oxidative stress is one of the general internal signals that affect mitochondria, and ROS in turn contribute to levels of oxidative stress (Duan et al., 1999; Ueda et al., 2002).

ROS and cysteine oxidation

In its reduced state, cysteine is a thiol; when oxidized it can form disulfide bonds or other structures. Proteins containing cysteine can thus have one conformation when reduced and another when oxidized. Either or both forms of the protein may be functionally important. Typically, high levels of ROS result in oxidation. A number of proteins have their activity regulated in this way (e.g. tyrosine phosphatases: Salmeen et al., 2003; van Montfort et al., 2003).

Materials and methods

Colonies of a single clone of *P. carnea* were cultured using standard methods (e.g. Blackstone, 1999; the same clone, P3, has been used extensively in previous investigations). For visualization of peroxides and stolon tips, colonies were grown on 15 mm diameter round glass cover slips. For studies of development, colonies were grown on 18 mm diameter round glass cover slips. Growth of the colonies was confined to one side of the cover slips by daily scraping of the reverse side with a razor blade. All experiments were carried out at 20.5 °C.

Hydrogen peroxide represents a major component of ROS under physiological conditions (Chance et al., 1979), and measures of H_2O_2 were taken using 2′, 7′-dichlorofluorescin diacetate (H_2DCFDA; see Blackstone, 2003). A 10 mM stock solution of H_2DCFDA was prepared in anhydrous DMSO. A day after feeding, colonies were incubated at 10 μmol l^{-1} for 1 h in the dark. Using a Hamamatsu Orca-100 cooled-CCD camera and a Zeiss Axiovert 135, fluorescence derived from the presence of peroxide was imaged for a representative polyp-stolon junction (excitation 450–490 nm, emission 515–565 nm).

Vitamin C (L-ascorbic acid) acts as a potent, soluble antioxidant in biological fluids. Vitamin C can scavenge both ROS and RNS (Carr & Frei, 1999). Methods were adapted from Jantzen et al.

(1998). A 10 mmol l^{-1} stock solution of vitamin C in seawater was prepared each day. NaOH was added to adjust the pH of the stock to that of seawater. Colonies were treated 6–8 h/days at a concentration of 100 μmol l^{-1} vitamin C in the dark for up to 60 days. These treated colonies were visually compared to similar-sized control colonies grown under the same conditions. To assess the effects of vitamin C on peroxides, naïve (i.e. previously untreated) colonies were incubated for 1–2 h in the dark in the same concentrations of vitamin C and H_2DCFDA as used above, and polyp-stolon junctions and stolon tips were imaged. Peripheral stolon tips were also imaged at visible wavelengths for a representative treatment and control colony.

Given the potential roles for ROS in signaling, it is worthwhile to ask what possible protein targets there may be. This cannot be directly addressed in *P. carnea*. To sample a range of proteins that may be involved in redox signaling in hydroids, the *Hydra magnipapillata* Ito expressed sequence tag (EST) data base was examined using BLAST. Using the translated database (tblastn), *H. magnipapillata* homologues of redox-regulated proteins from other species were identified.

Results and discussion

The mitochondrion-rich EMCs of the polyp-stolon junctions are the primary source of peroxides in normally growing colonies of *P. carnea* (Fig. 1; see Blackstone et al., 2004). Hydrogen peroxide diffuses easily through membranes. Gastrovascular fluid velocities near peripheral polyps can exceed 500 μm/s, while fluid velocities near central polyps are considerably faster (Van Winkle & Blackstone, 1997). Hence, H_2O_2, other ROS, and perhaps other redox-sensitive molecules from the polyp-stolon junctions may circulate widely throughout the colony. Figure 1 suggests that stolons normally contain little peroxide, while stolon tips exhibit somewhat higher concentrations. A number of additional experiments support these observations. Due to space constraints these are only briefly summarized here:

(1) Tips of short, centrally located stolons exhibit a higher concentration of peroxide than tips of longer, peripheral stolons.

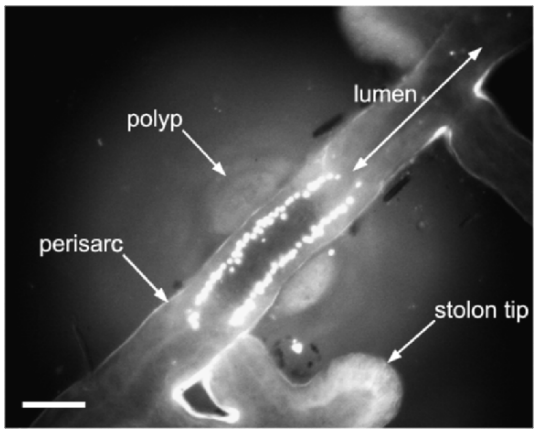

Figure 1. Fluorescent micrograph of the polyp-stolon junction and surrounding stolons in a living colony of *P. carnea* treated with H_2DCFDA. The colony is viewed from beneath using an inverted microscope. Various single-celled organisms produce fluorescence or dark areas outside the perisarc (e.g. near the polyp). The perisarc itself exhibits some fluorescence particularly in areas where it is thickened. The tissues of the stolon tip and of the polyp show some fluorescence. Intense fluorescence of living hydroid tissue is limited to mitochondrion-rich EMCs at the polyp-stolon junction. Scale, 50 μm; to enhance contrast, image is stretched for 'best fit'.

(2) Exogenous peroxide, added at physiological doses, increases the levels of peroxide in stolon tips.
(3) *Eirene viridula* (Peron & Lesueur), an extreme runner-like colonial hydroid, exhibits higher levels of peroxide in stolons and stolon tips than *P. carnea*.

Treatment with 100 μmol l^{-1} vitamin C has striking effects on colony growth (Fig. 2). Peripheral stolon tips become much shorter and branch more frequently. Outward growth of the stolons and hence the colony is greatly slowed by vitamin C and more sheet-like growth of the colony results. Similar effects on growth are achieved when ROS are diminished by inhibiting mitochondrial electron carriers (complex I) upstream of the site of reactive oxygen formation or by uncoupling oxidative phosphorylation (Blackstone, 1999, 2003). The opposite phenotypic effect – runner-like growth with little branching and relatively elongated stolon tips – is achieved when ROS are upregulated by inhibiting electron carriers (complex III and IV) downstream of the site of reactive oxygen formation (Blackstone, 1999, 2003). Inbred lines of *P. carnea* selected for sheet-like or

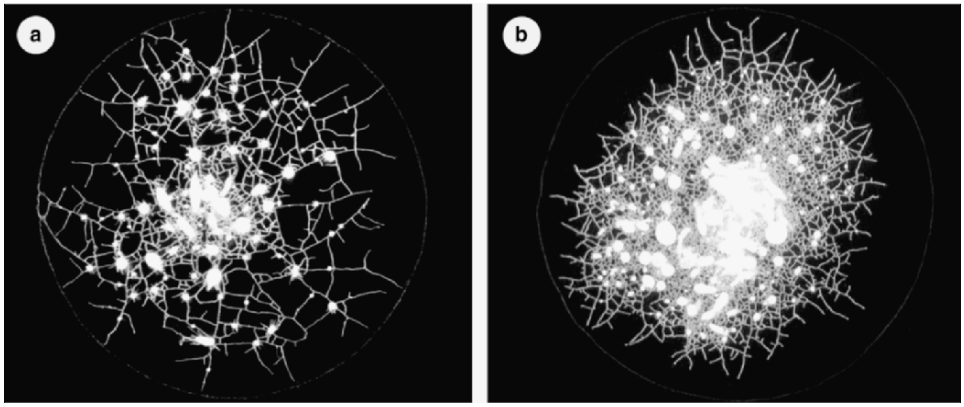

Figure 2. Images of colonies of *P. carnea* growing on 18 mm diameter glass cover slips. Polyps are bright and circular; stolons are darker and web-like. (a) Control, image taken after 30 days treatment. (b) Treated with 100 μmol l^{-1} vitamin C for 6–8 h/days; image taken after 60 days treatment.

runner-like growth also differ in peroxide levels in the predicted direction: polyp-stolon junctions of runner-like colonies exhibit higher levels of ROS than those of sheet-like colonies (Blackstone et al., 2004).

In naïve (i.e. previously untreated) colonies, vitamin C treatment has an immediate effect on peripheral stolon tips, causing them to regress within 2 h of treatment (Fig. 3). The longest peripheral stolons (top panels of Fig. 3) are most strikingly affected by this treatment. This effect disappears after a colony is treated for several weeks and has developed a sheet-like form (e.g. Fig. 2b). In treated naïve colonies, the regression of peripheral stolons occurs amidst a large flux of peroxide (Fig. 4). Similar high levels of peroxide are not seen in healthy stolon tips, including central stolon tips of treated colonies and both central and peripheral stolon tips of untreated colonies. Regardless of the specific trigger for the regression, preliminary data further suggest that regressing stolon tips generally exhibit a large peroxide flux. Stolon regression may be an active process related to the generation of peroxides in the regressing stolons themselves. This process is rapid (initiated within hours of environmental stimuli), allowing colonies to adapt their growth on ecologically relevant time scales (Blackstone, 2003).

ROS may thus be involved in two distinct aspects of signaling in hydroid colonies. The first involves carrying signals from mitochondrion-rich EMCs of polyp-stolon junctions to growing stolons and stolon tips. Manipulations of the redox state of mitochondria support such a role for ROS (Blackstone, 2003). Experiments with the antioxidant vitamin C provide further support. On the other hand, experiments with glutathione ethyl ester (a form of reduced glutathione that can be taken up by cells) have no detectable effect. Redox-sensitive molecules other than ROS may carry signals from polyps or polyp-stolon junctions to growing stolons and stolon tips. ROS, particularly peroxides, also seem to be implicated in stolon regression. Here, the occurrence of high levels of peroxides and cell and tissue death seems more than coincidental.

Using *H. magnipapillata* as a model, several exemplars of potential redox-regulated proteins are presented in Table 1. The primary mechanism of regulation is the reversible oxidation of a cysteine residue. For instance, the putative protein tyrosine phosphatases of *H. magnipapillata* exhibit a characteristic PTP loop signature – VHCSGGVGRT – which contains a catalytic cysteine (van Montfort et al., 2003). When this cysteine is oxidized by H_2O_2, tyrosine phosphatases are inhibited. Target protein dephosphorylation is suppressed, and signal transduction pathways mediated by tyrosine kinases are optimally activated (Salmeen et al., 2003). On the other hand, peroxiredoxins directly mediate H_2O_2 signaling via their antioxidant function. When H_2O_2 is reduced, the catalytic cysteine residue is oxidized. At low concentrations of peroxide, a disulphide bond will form with the

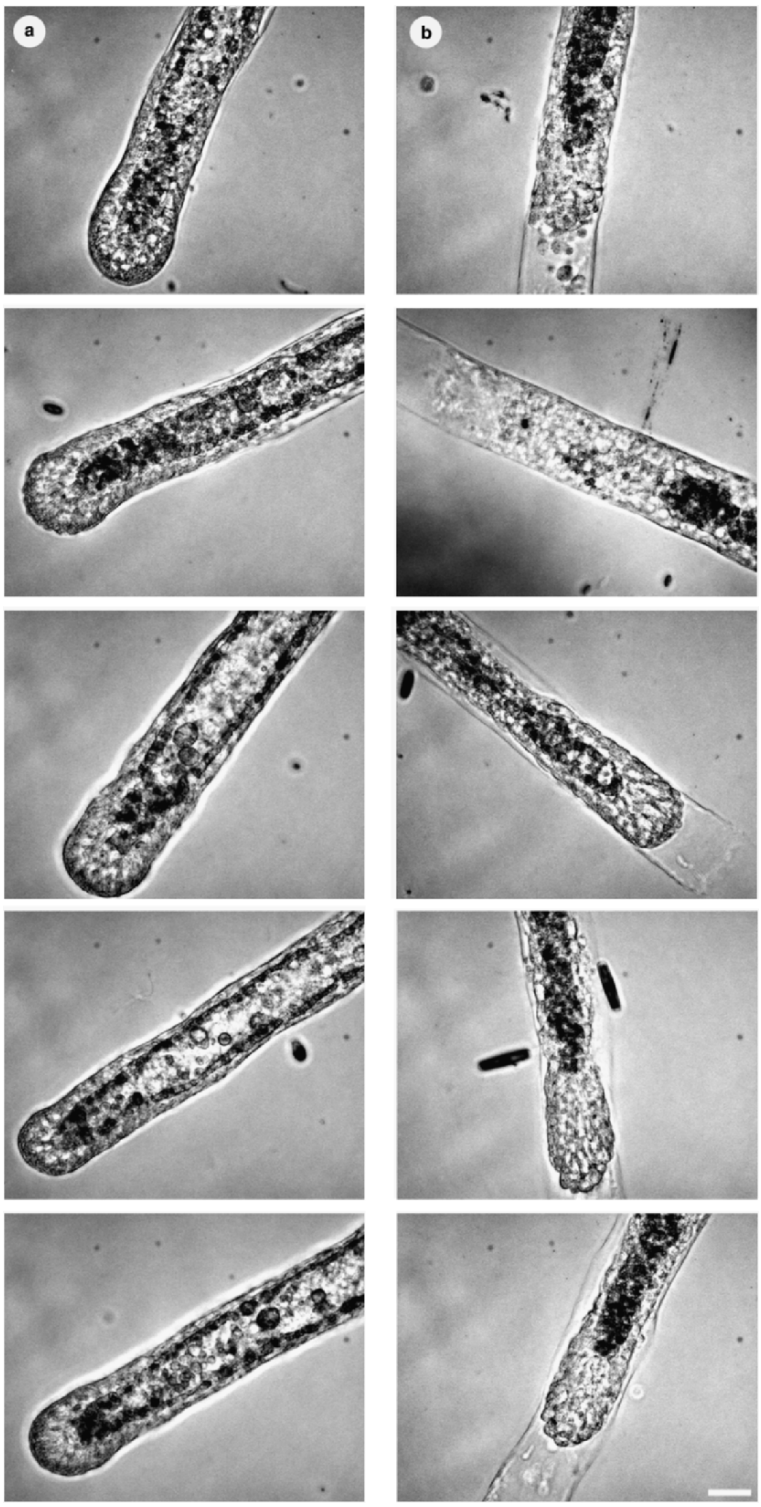

Figure 3. Micrographs of peripheral stolon tips from two previously untreated colonies of *P. carnea*. Images of tips from the longest peripheral stolons are shown in the top panels. (a) Tips from a control colony. (b) Tips from a colony treated for 2 h with 100 μmol l^{-1} vitamin C. Scale, 25 μm; to enhance contrast, images are stretched for 'best fit'.

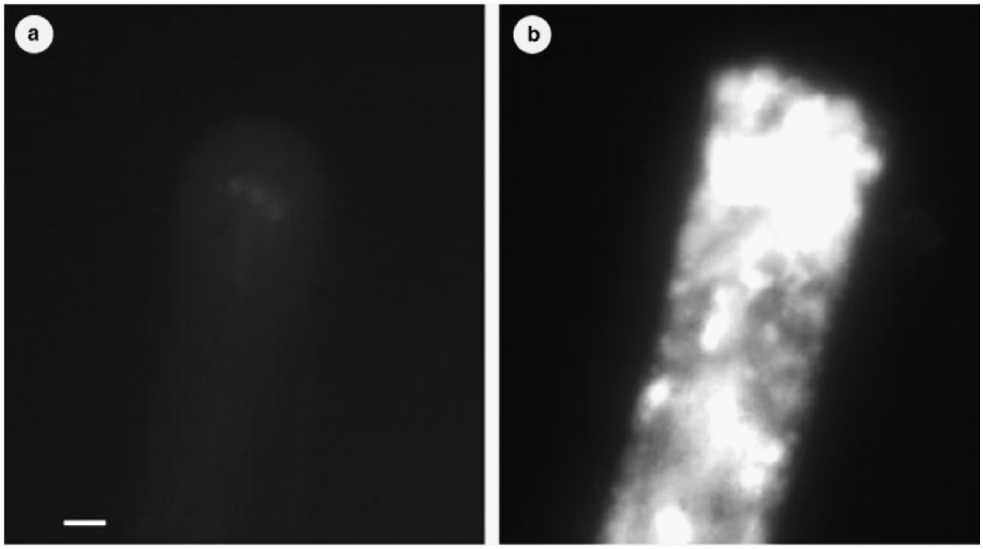

Figure 4. Fluorescent micrographs of peripheral stolon tips similar to those in Figure 3 except treated with H_2DCFDA. (a) Tip from a control colony. (b) Tip from a colony treated for 2 h with 100 μmol l^{-1} vitamin C. Scale, 10 μm.

cysteine from the other subunit of the homodimer. Thioredoxin subsequently reduces the disulfide bond. However, at high concentrations of peroxide, the catalytic cysteine may react with H_2O_2 again to form higher oxidation products, which cannot be reduced by thioredoxin. Such overoxidation of peroxiredoxins then leaves peroxide available to participate in other signaling functions (Georgiou & Masip, 2003). In zinc finger proteins such as SAG, at least two cysteines are necessary to form the zinc finger structure. The thiol/disulfide equilibrium interacts with the properties of zinc to produce potentially sophisticated redox behavior (Duan et al., 1999; Lee & Maret, 2001). Such redox-mediated signaling pathways seem to a shared feature of eukaryotes, and the data from *H. magnipapillata* indicate that cnidarians are not exceptions to this general rule.

Conclusion

Given the numerous experiments yet to be carried out, conclusions at this time must be considered tentative. Previous experiments suggest that feeding-related metabolic demand affects the redox state of mitochondrion-rich EMCs at polyp-stolon junctions, altering ROS formation (Blackstone, 2001, 2003). The mitochondrion-rich EMCs at polyp-stolon junctions likely determine the level of ROS throughout the colony under most circumstances. If these mitochondria emit high levels of ROS, an oxidizing environment will prevail throughout the gastrovascular fluid. Polyps or polyp-stolon junctions may also release various as yet unspecified redox-sensitive molecules into the gastrovascular fluid. Stolons and stolon tips may be sensitive to the gastrovascular environment. Perturbations of this environment may lead to rapid and active regression of stolons in which large quantities of ROS are generated by the stolons themselves. In this way, signals from polyp-stolon junctions may affect colony-level form, allowing colonies to adapt their form to environmental changes on an ecologically relevant time scale. Three specific hypotheses to explain the effects of ROS on stolons are proposed:

(1) ROS, particularly superoxide, produced by mitochondria at polyp-stolon junctions may interact with the vasodilator nitric oxide. Under some circumstances, this may lead to diminished levels of nitric oxide in peripheral stolons. These stolons then close and initiate active regression mediated by locally produced peroxides.

Table 1. Summary of several BLAST searches for potential redox-regulated proteins using the translated *H. magnipapillata* expressed sequence tag data base

Protein	EST/cDNA[a]	Identities(positives)/total[b]	Query species	References
Tyrosine phosphatase	taa44h06.x3	69 (110)/273 [yeast] 85 (126)/216 [human]	*Saccharomyces cerevisiae* *Homo sapiens*	Goffeau et al. (1996) GenBank
Tyrosine phosphatase	taa14d12.y1	61 (103)/179	*Caenorhabditis elegans*	Kamath et al. (2003)
Tyrosine phosphatase	taa28e05.y1	59 (90)/181	*Amsacta moorei entomopoxvirus*	Bawden et al. (2000)
SAG	taa17d11.y1	11 (19)/28	*Homo sapiens*	Duan et al. (1999)
Thioredoxin	taa11b05.y1	31 (48)/83	*Drosophila melanogaster*	Salz et al. (1994)
Y Box pro 3	taa15e05.y1	72 (79)/89	*Mus musculus*	Duh et al. (1995)
Peroxiredoxin	taa01d10.x1	65 (109)/175	*Salmonella typhimurium*	Wood et al. (2002)
Peroxiredoxin	taa05a05.y1	108 (136)/199	*Drosophila melanogaster*	Adams et al. (2000)
Peroxiredoxin	tab34c05.x1	112 (130)/182	*Homo sapiens*	Wagner et al. (2002)

[a] Each search yielded 5–80 similar ESTs; only the most similar is listed here.
[b] Identities are identical amino acids; positives include identities as well as chemically similar amino acids. Ratios are calculated only for sequences similar enough to align.

(2) Levels of ROS emitted by mitochondria at polyp-stolon junctions may affect the redox state of cells lining the peripheral stolons. Certain perturbations of the redox state of stolonal cells leads to programmed cell death and active regression mediated by locally produced peroxides.
(3) Whether produced by mitochondria or other cellular sources, ROS may regulate protein-signaling cascades that lead to stolon tip growth or branching as well as polyp initiation. Several putative candidates for such signaling cascades are discussed. Other candidates include vascular endothelial growth factor (VEGF, see Bridge, 2004, this volume).

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Variations on a theme? Polyp and medusa development in *Podocoryna carnea*

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Key words: emx, forkhead, NK-2, Podocoryne, Cnidaria

Abstract

The life cycles of many cnidarian species are notable for including two stages with very different morphologies – sessile polyp and swimming medusa. Cnidarians thus provide an opportunity to study the developmental bases of differences in body organization without the need to compare organisms of different taxa. Information about the two life cycle stages suggests the following questions about differences in their development. (1) Are the mouth and tentacle-bearing region (bell margin) specified using any of the same molecular mechanisms as in the polyp? (2) Has the oral-aboral axis of the medusa been truncated relative to that of the polyp by elimination of molecular processes specifying aboral tissue identity? (3) Is the elongated region between the hydrozoan medusa mouth and tentacle ring (the manubrium) patterned using processes that pattern the entire oral-aboral axis in the polyp? We describe how data on their expression of *FoxA*, NK-2, and Emx genes during polyp and medusa development, together with reagents targeting specific signaling pathways, could be used to address these questions. We have isolated portions of a *FoxA2* homologue, an NK-2 gene, and two Emx genes from *Podocoryna carnea* Sars, an experimentally tractable hydrozoan with both polyp and medusa stages. Phylogenetic analyses indicate that the two *P. carnea* Emx genes are the result of a gene duplication.

Introduction

In many species within the phylum Cnidaria, a sessile polyp stage is followed by formation of a morphologically very different free-swimming medusa stage. These life cycle stages differ radically in the position of structures along the oral-aboral body axis and in overall body proportions. The developmental bases of these morphological differences remain unclear. Although the biology of the polyp has received considerable study (Miller & Ball, 2000; Frank et al., 2001; Galliot & Schmid, 2002; reviewed in Bode, 2003; Holstein et al., 2003), less is known about the development of the medusa (reviewed in Galliot & Schmid, 2002). Here we consider existing data on polyp development and outline a series of questions about developmental differences underlying the morphological differences between the polyp and the medusa stages. We describe ways to address these questions by comparing expression of relevant genes during polyp and medusa development. Finally, we present data on the genes of interest.

The phylum Cnidaria includes species with a polyp and no medusa, species with both life cycle stages, and species with a medusa stage and no polyp. Phylogenetic analyses of both morphological and molecular data sets suggest that the class Anthozoa, whose members possess only the polyp stage, is basal within the phylum, and that the

classes possessing a medusa stage form a monophyletic group (Schuchert, 1993; Bridge et al., 1995; Kim et al., 1999; Collins, 2002). The simplest interpretation of this result is that the polyp stage evolved before the medusa stage and that the medusa arose through major modification of polyp development. It should be noted, however, that loss of the medusa stage in an ancestor of the class Anthozoa would have involved only one additional evolutionary event. Comparative developmental data may provide further insight into the question of which stage arose earlier and how the development of one stage might have been modified to produce the other.

Polyp development

A substantial body of research exists on developmental processes in cnidarian polyps. A polyp consists of a tube with a mouth and a ring of tentacles at one end, colloquially referred to as a 'head' (Fig. 1). The basis of the polyp oral-aboral axis has been the subject of extensive study, particularly in members of the genus *Hydra*. In *Hydra*, morphogenesis and patterning occur continuously, with cells dividing in the body column and being displaced toward the ends of the animal, where they differentiate (Campbell, 1967). *Hydra* axial patterning has been well described at the tissue and cell level, by means of grafting experiments and other tissue fate manipulations (for review, see Bode & Bode, 1984). Such experiments have established that tissue of the hypostome region at the oral end possesses signaling properties; it is capable of inducing a second axis when grafted into the body column of a host animal (Browne, 1909; Webster & Wolpert, 1966; Broun & Bode, 2002). Much of the work on *Hydra* axial patterning has involved polyps developing by the asexual process of budding. Budding occurs by evagination of a circular field of tissue, with both endoderm and ectoderm evaginating (Otto & Campbell, 1977). The future oral end of the developing bud acquires the ability to induce a secondary axis before tentacles form (Broun et al., 1999).

Medusa development

The medusa is the mobile, sexually reproducing stage of the cnidarian life cycle in those taxa possessing it. The medusa consists of a bell (Fig. 1), whose muscular contractions power swimming. Tentacles, with a somewhat different morphology from those of polyps, are located around the bell margin in most medusae. Sensory structures, such as photoreceptors and gravity-sensing statocysts, are also found along the bell margin. In members of class Hydrozoa, the mouth is present at the end of a tube hanging down from the bell, known as the manubrium (Hyman, 1940). In *Podocoryna carnea*, four short oral arms, tentacle-like outgrowths of the manubrium lip, are present around the mouth (Bouillon, 1985). The gastric cavity

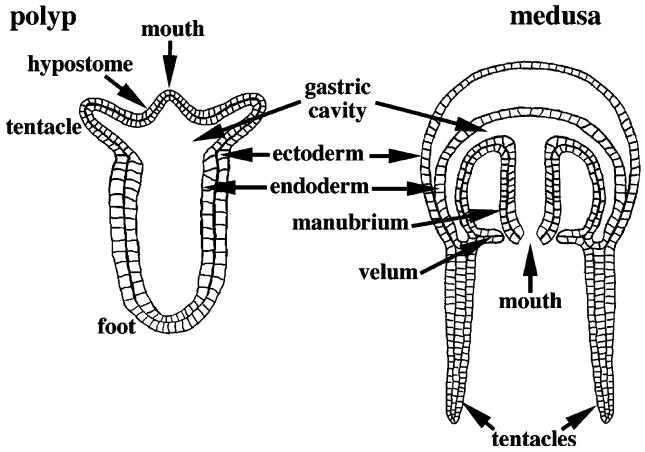

Figure 1. Diagram of a *Hydra* polyp and a hydrozoan medusa. In *Hydra* the region between the mouth and tentacle ring is known as the hypostome.

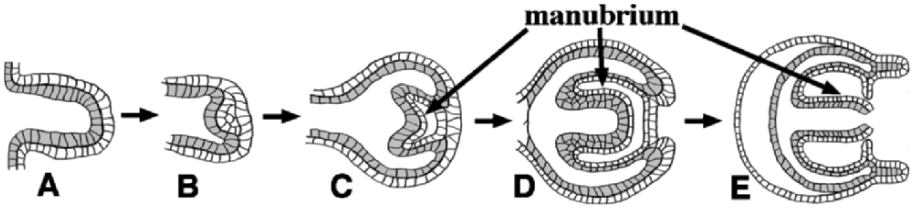

Figure 2. Development of a hydrozoan medusa.

extends from the base of the manubrium into the bell in the form of tubular radial canals.

In most members of the class Hydrozoa, medusa formation proceeds by budding from the side of the polyp (Hyman, 1940; Boelsterli, 1977), a process whose early stages are similar to the formation of polyps by budding. Like polyp budding, medusa budding initially involves evagination of both endoderm and ectoderm (Fig. 2A). However, during medusa formation, ectodermal epithelial cells at the tip of the bud proliferate (Fig. 2B) and form an internal cavity. One side of this cavity evaginates to produce the manubrium (Fig. 2C and D). Completion of development involves opening of the cavity, growth of tentacles, and constriction of the tissue attaching the medusa to the parent polyp (Fig. 2E). Both future mouth and future bell margin tissue originate from the distal end of the early-stage evaginating bud (Fig. 2A and B). Expression of genes at the distal end early during budding would thus affect both the mouth and the tentacle-bearing bell margin, even though the mouth and tentacles are farther apart in the adult medusa than in the polyp.

Understanding of medusa development has been complicated by the fact that medusa development differs within the Cnidaria. In examined members of the class Cubozoa, the medusa is produced by metamorphosis of the polyp (Werner et al., 1971). The polyp tentacles become sensory structures along the margin of the medusa bell. The medusa mouth forms at the location of the former polyp mouth. In the orders Semaeostomae, Rhizostomae, and Coronatae, the medusa is produced by the process of strobilation (Brusca & Brusca, 2002). Strobilation begins with polyps undergoing morphological change, during which their tentacles are reabsorbed. This is followed by horizontal fission at the oral end which releases immature medusae (Spangenberg, 1965). As in Cubozoa, the oral end of the polyp becomes the oral end of the medusa. The questions and experiments described here focus on development of a hydrozoan medusa, in part because of the advantages of being able to compare polyp and medusa formation by budding. However, the questions outlined apply to medusa development in the other classes as well.

Differences between polyp and medusa development

Comparison of polyp and medusa budding suggests three initial questions about the differences in their development.

(1) Does specification of the mouth and tentacle ring of the medusa involve some of the same molecular mechanisms as specification of the mouth and tentacle ring of the polyp?

In the adult medusa, the mouth and tentacle-bearing bell margin are relatively far apart. However, both regions may show developmental parallels to the polyp head. Medusa development in all cnidarian taxa with medusae suggests that this may be the case. For instance in the Hydrozoa, tissue at the distal end of the early medusa bud forms the mouth, manubrium, and bell margin, as tissue at the distal end of the early polyp bud forms the mouth and tentacles.

The question of whether there are parallels in development of the mouth and tentacle zone in polyps and medusae can be addressed by examining expression in medusae of selected genes known to be expressed at the oral end of the polyp in *Hydra* or other taxa (Fig. 3). One such gene is *Hydra vulgaris HyWnt*, which encodes a member of the Wnt/wingless family of secreted signaling proteins. *HyWnt* is expressed in tissue immediately adjacent to the mouth (Hobmayer et al., 2000). The *Hydra vulgaris* gene *budhead*, a homologue of the mouse gene *FoxA2*, is expressed in the endo-

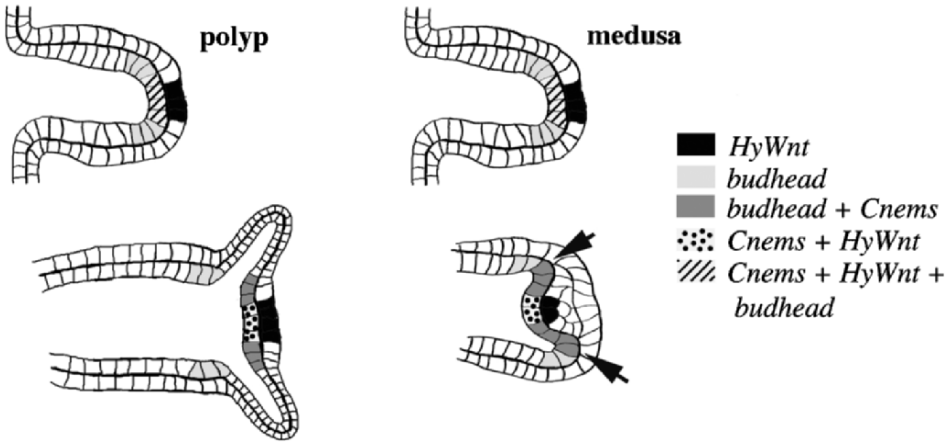

Figure 3. Expression of *Hydra budhead* and *HyWnt* and *Hydractinia Cn-ems* during budding in polyps and predicted expression of homologues in medusae. Arrows indicate where the medusa tentacles will develop.

derm at the base of the hypostome (Martínez et al., 1997). Expression of both *HyWnt* and *budhead* begins early in polyp budding, by the beginning of bud evagination. *Cn-ems*, a *Hydractinia symbiolongicarpus* homologue of the *Drosophila* homeobox gene *ems*, is expressed in the endoderm of the polyp hypostome, over a broader area than *HyWnt* (Mokady et al., 1998). Because these genes are expressed in an overlapping fashion at the distal end of the polyp, information about their expression in the developing medusa should allow a detailed comparison of polyp and medusa formation. One possibility is that the future mouth region of the developing medusa, like that of the polyp, has inductive properties. In this case, tissue which would go on to form the medusa tentacle-bearing region (bell margin) might show gene expression characteristic of the polyp tentacle zone, but only in early developmental stages, when it is close to the future mouth.

(2) Has the oral-aboral axis of the medusa been truncated relative to that of the polyp by elimination of molecular processes specifying aboral tissue identity?

The oral-aboral axis of the medusa is less elongated than that of the polyp. While a mouth must be specified at one end in both polyp and medusa, the medusa does not possess a morphologically well-defined aboral end as does the polyp. Loss during the evolution of medusa development of processes specifying aboral tissue in the polyp would account for some of the gross differences between polyp and medusa organization.

This question may be addressed by determining the expression pattern of the homeobox gene *CnNK-2* in the early stages of developing medusae. In *Hydra vulgaris*, *CnNK-2* expression is correlated with aboral tissue identity in both normal animals and those in which tissue fate has been experimentally altered. *CnNK-2* is expressed in prospective polyp bud aboral tissue starting before this tissue moves into the evaginating bud (Grens et al., 1996). If medusae indeed lack the processes used to specify aboral tissue in polyps, we might expect *Podocoryna carnea NK-2* expression to be absent from the base of the developing medusa bud (Fig. 4B), although present in developing polyps.

(3) Is the manubrium axis established and maintained using the same mechanisms as the polyp oral-aboral axis?

The manubrium, particularly in the Hydrozoa, is an elongated, tube-like structure with the mouth at one end. It is possible that polyp oral-aboral axial patterning machinery has been co-opted to pattern this structure. If this is true, we would expect genes associated with formation of the distal structures of the polyp to be turned on initially to specify the future mouth and bell margin and genes associated with aboral axial patterning in the polyp to be turned on later, during manubrium growth.

This issue can be addressed by determining the expression pattern of *CnNK-2* in the late stages of

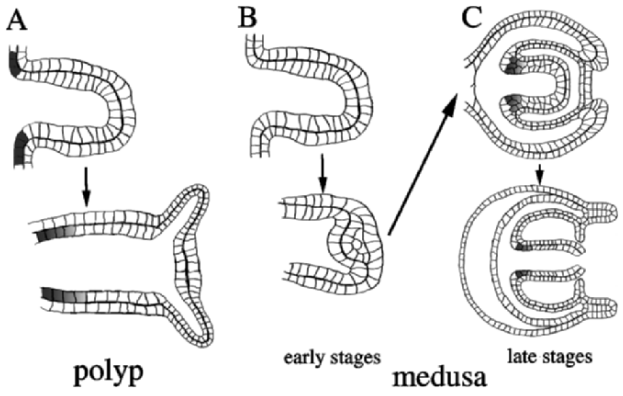

Figure 4. (A) Expression pattern of *CnNK-2* during *Hydra vulgaris* polyp budding. (B) and (C). Expression of an *CnNK-2* homologue predicted if *CnNK2* is not involved in development of the medusa aboral end (B) but is involved in patterning of the manubrium (C).

developing medusae. In *Hydra vulgaris* polyps, *CnNK-2* expression is correlated with tissue positional value along the oral-aboral axis (Grens et al., 1996). If *CnNK-2* expression in the manubrium is similar to that in the polyp, with expression graded and highest at the aboral end (Fig. 4C), this will provide strong evidence that manubrium patterning and polyp patterning share molecular components.

Material and methods

Genomic and cDNA were produced from the *Podocoryna carnea* strain P2, originally collected from the Yale Peabody Museum Field Station in Connecticut. Genomic DNA was extracted from medusae using a Qiagen DNeasy Tissue kit. Initial fragments of genes were amplified from genomic DNA using touchdown PCR with degenerate primers. Primers were designed based on the conserved regions of genes homologous to those of interest. Additional portions of the *P. carnea* genes were obtained using the splinkerette PCR walking approach (Devon et al., 1995) and using RACE PCR (Frohman & Martin, 1989). For RACE PCR, RNA was extracted both from medusae and from colonies grown on microscope slides using a QiagenRNeasy Mini kit. Poly A^+ RNA was isolated using a Qiagen Oligotex mRNA Mini kit.

To examine the relationships of putative *P. carnea* Emx and NK-2 genes to other genes, phylogenetic analyses were performed using predicted homeodomain amino acid sequences. Analyses for Emx sequences included *Hydractinia* Cn-ems and mouse, amphioxus, and *Drosophila* Emx sequences. Accession numbers for the sequences used are Cn-ems: CAA72534, Emx1: XM 132640, Emx2: NM 004098, Evx1: NP_031992, Hoxb3: P09026, AmphiEmxA: AAF76327, AmphiEmxB: AAK93792, ems: P18488, and E5: AAF54997. Analyses for NK-2 sequences included *Hydra* CnNK-2 and mouse and *Drosophila* NK-2 sequences. Accession numbers for the sequences used are Nkx2.1: AAC35350, Nkx2.2: AAA79303, Nkx2.3: AAD38415, Nkx2.4: AAG35618, Nkx2.5: AAC97934, Nkx2.6: AAC15674, Nkx3.1: AAC52956, Msx1: AAH16426, Dlx1: AAB40900, NK-2/vnd: P22808, scarecrow: AAF26436, tinman: AAF55890, and bagpipe: P22809. Phylogenetic analyses were performed using maximum parsimony methods with PAUP* 4.0 (Swofford, 2003). The protpars matrix of PAUP* 4.0 was used to weight amino acid substitutions. To obtain bootstrap values, 1000 bootstrap pseudoreplicates were performed with branch and bound searching.

Results

We have cloned and sequenced portions of *budhead* from *Podocoryna carnea* (Fig. 5). Both the nucleotide and the predicted amino acid sequences from *P. carnea budhead* are very similar to those from *Hydra vulgaris*. The similarity is particularly

Figure 5. (A) Sequence alignment of Budhead proteins for *Podocoryna carnea* (Pc) and *Hydra vulgaris* (Hv). Underlined sequence marks the fork head DNA-binding domain. Shaded box corresponds to a transcriptional activation domain. (B) Sequence alignment including the homeodomains of Emx proteins for *P. carnea*, *Hydractinia symbiolongicarpus* (Hs) and *Drosophila melanogaster*(Dm). The non-Emx protein Antennapedia is included for comparison. The arrow indicates the presence of an intron in the *P. carnea* and *H. symbiolongicarpus* sequences. (C) Sequence alignment of the homeodomains of NK-2 proteins for *P. carnea* and *H. vulgaris*, together with Antennapedia. Dots indicate identity and hyphens indicate gaps.

high at the 5′ end and in the regions encoding the fork head domain and the transcriptional activation domain located downstream from the fork head domain (Fig. 5A).

We have also cloned portions of two putative *P. carnea* homologues of *Hydractinia symbiolongicarpus Cn-ems*, designated *emsa* and *emsb* (Fig. 5B). The genes each include an intron within the homeobox region, at the same location as the intron present in the homeobox region of *Cn-ems*. These introns are of different sizes – 382 nucleotides long for *emsa* and 545 nucleotides long for *emsb*. For *emsa*, a region beginning 5′ of the homeobox and extending to the 3′ end of the gene has been cloned and sequenced. For *emsb*, the entire coding region of the gene has been cloned and sequenced. The genes show differences at the nucleotide level throughout the regions sequenced. Phylogenetic analyses using the predicted sequences of the emsa and emsb homeodomains group *emsa* and *emsb* together with *Hydractinia Cn-ems* (Fig. 6). Analyses provide strong support for placement of *Podocoryna emsa* and *emsb* with the Emx genes (Fig. 6).

Part of an apparent *P. carnea Cn-NK2* homologue has also been cloned and sequenced (Fig. 5C). A 621 bp intron is present within the homeobox region, and an additional intron is located approximately 216 nucleotides upstream of the homeobox. Phylogenetic analyses using the predicted homeodomain sequence of *Podocoryna NK-2* provides strong support for its orthology with *Hydra Cn-NK2* (Fig. 7). Analyses place these two cnidarian genes with mouse and *Drosophila* NK-2 genes (Fig. 7).

Discussion

In this paper, we outline three questions about the relationship between cnidarian polyp and medusa development. Working with the hydrozoan *Podocoryna carnea*, we have cloned portions of

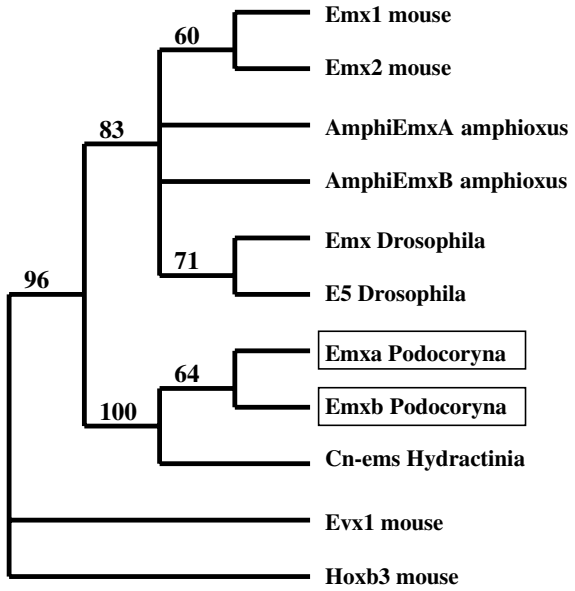

Figure 6. Phylogenetic tree including *P. carnea emsa* and *emsb*, based on homeodomain amino acid sequences. A strict consensus of trees from maximum parsimony bootstrap pseudoreplicates is shown. Numbers shown are bootstrap values. For the most parsimonious tree, CI is 0.78 and RI 0.75.

developmental regulatory genes whose expression patterns should be particularly useful in addressing these questions. These genes are members of FoxA, Emx, and NK-2 families. A Wnt/wingless gene, which would also be informative, remains to be isolated. *P. carnea* appears to possess two homologues of *Drosophila ems*, although only one *ems* homologue has been identified from *Hydractinia symbiolongicarpus*, another member of the Hydractinidae (Mokady et al., 1998). Comparison of the *P. carnea ems* sequences to those of Emx genes from other organisms suggests that the *P. carnea* ems genes have arisen during the evolution of the Cnidaria rather than through a gene duplication in a common ancestor of cnidarians and other phyla (Fig. 6). *H. symbiolongicarpus Cn-ems, P. carnea emsa* and *P. carnea emsb* all include an intron at the same location within homeobox. Other Emx genes for which intron location has been determined, including genes from human, mouse, amphioxus, *Drosophila,* and *C. elegans*, lack an intron at this position (Williams & Holland, 2000). This shared feature

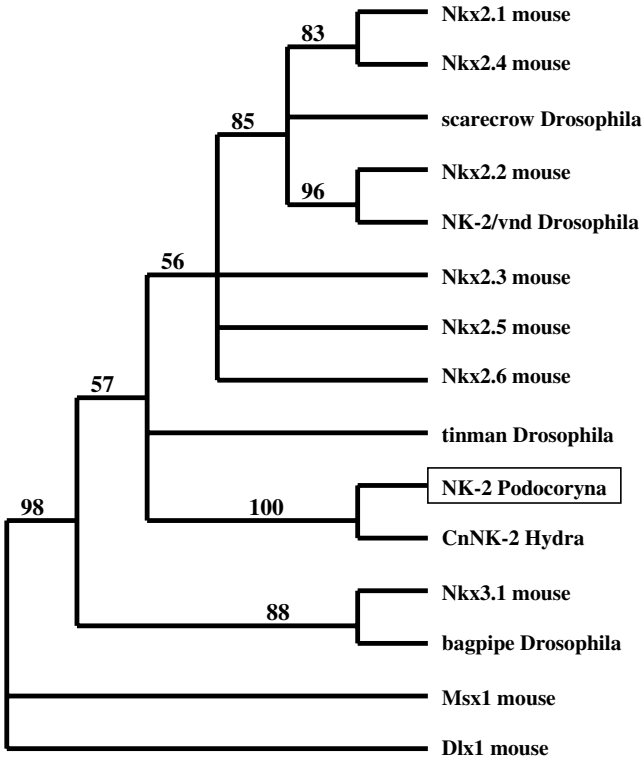

Figure 7. Phylogenetic tree including *P. carnea NK-2* based on homeodomain amino acid sequences. A strict consensus of trees from maximum parsimony bootstrap pseudoreplicates is shown. Numbers shown are bootstrap values. For the most parsimonious tree, CI is 0.72 and RI 0.72.

provides further evidence that the two *P. carnea* ems genes arose during the course of cnidarian evolution.

Molecular studies of medusa development have focused primarily on differentiation of the striated muscle (epithelial-muscular cells of ectodermal origin) lining the underside of the bell (for review, see Galliot & Schmid, 2002). However, recent data on three genes which are expressed in both polyp and medusa stages begin to answer the first question we raise here – whether there are parallels between development of the polyp oral end and the medusa mouth and bell margin. These results indicate that such developmental parallels do exist. One gene expressed at the oral end of the polyp is the T-box gene *Brachyury*. In adult polyps of *P. carnea*, *Brachyury* is expressed in the endoderm in the upper portion of the hypostome (Spring et al., 2002). During development of *P. carnea* medusae, *Brachyury* is expressed in the manubrium from the early stages of its formation. As development proceeds, *Brachyury* expression in the manubrium is localized to tissue adjacent to the mouth (Spring et al., 2002). The *P. carnea* orphan Hox gene *Cnox2-Pc* is also expressed at the oral end of the adult polyp, but in the ectoderm of a more restricted region than *Brachyury* (Masuda-Nakagawa et al., 2000). In early stages of medusa formation, *Cnox2-Pc* is expressed in ectoderm at the tip of the medusa bud, the tissue which will give rise to the bell margin. Expression is seen only in this region even after manubrium formation begins (approximately the stage shown in Fig. 2C); *Cnox2-Pc* is not expressed near the future mouth in the developing medusa manubrium (Masuda-Nakagawa et al., 2000). Finally, like *Cnox2-Pc*, the MADS-box gene *Mef2* is expressed in ectoderm immediately adjacent to the polyp mouth and in the ectoderm of the distal end of the developing medusa (Masuda-Nakagawa et al., 2000). Thus in the developing *P. carnea* medusa, these three genes expressed at the oral end of the polyp are all expressed in either the prospective mouth or the prospective bell margin. Among the three transcription factor genes, it is notable that the two expressed in the polyp ectoderm are the ones expressed in the future medusa bell margin.

In a polyp formed by budding, the mouth and the region around it develop from tissue at the distal end of the early bud. In a medusa formed by budding, the mouth, the manubrium, and the bell margin all develop from the distal end of the early bud. It would therefore be understandable for tissue of the developing medusa bell margin to express some genes which are characteristic in the polyp of tissue near the mouth. At later stages of medusa development, as the prospective mouth and bell margin are spatially separated, gene expression in these tissues may come to differ. Characterizing the expression of genes such as *P. carnea Wnt*, *budhead, emsa,* and *emsb* will provide further insight into the relationship between polyp and medusa development.

Development of the medusa bell margin is of particular interest because it bears on the evolution of eyes. In cnidarians, the polyp stage lacks photoreceptors, while many medusae possess them. When present, photoreceptors, as well as gravity-sensing structures, are located on the bell margin (Hyman, 1940). The position of sensory structures far from the mouth might seem to indicate a substantial difference between body organization in medusae and bilaterians such as vertebrates and *Drosophila*. However, detailed comparison of polyp and medusa development provides insight into this difference. Thus understanding the relationship between polyp and medusa development should also elucidate the relationships between cnidarian development and that of other phyla.

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The development and evolution of hydrozoan polyp and colony form

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Key words: Hydrozoa, Cnidaria, colony, development, evolution

Abstract

Hydrozoans represent an extremely diverse group of mostly colonial forms. Despite this tremendous diversity, many of the morphological differences between hydrozoan species can be attributed to simple changes in the relative position of regions/structures along the axes of the polyp and the stolon or hydrocaulus from which polyps bud. Many genes have been implicated in the specification of positional information along the axis of the polyp. Knowledge from these studies in *Hydra*, and from comparative studies in *Hydractinia* polyp polymorphs, suggests that evolutionary changes in the regulation of axial patterning genes may be a prominent mechanism underlying hydrozoan evolution. Despite the paucity of interspecies comparative expression information, hypotheses can be formulated about the role of developmental regulatory genes in hydrozoan evolution from information available from *Hydra*.

Introduction

Hydrozoa is noted for its remarkable diversity in polyp and colonial forms. Confined to a simple tube-within-a-tube structure characteristic of the cnidarians, where a single layer of ectoderm encloses a single layer on endoderm, hydrozoans display a wide range of morphologies reflecting their diverse life histories, habitats, and developmental programs. A simple hydra polyp, an encrusting hydrocoral, and a complex pelagic siphonophore bear little resemblance to each other, yet the seemingly dramatic differences among these and other hydrozoan species can be attributed, in large part, to simple changes in the axial patterning of the polyps and the colony. The proper regulation of axial patterning in development is thus key to a species attaining its characteristic morphology. Furthermore, evolutionary changes in the regulation of axial patterning processes are likely prominent mechanisms underlying hydrozoan diversity.

Polyp morphology and diversity

A hydrozoan polyp consists of an outer ectoderm layer and an inner endodermally derived gastrodermis. The oral region consists of a hypostome with a mouth and tentacles, and the body column is a region that may bear gonophores or, in the case of *Hydra*, buds other polyps. The base of the polyp possesses specialized structures that adhere to the substrate in solitary forms; in colonial forms, the structures from which polyps bud are referred to as stolons if attached to the substrate (in encrusting forms), and hydrocauli if upright (in arborescent forms). Morphological differences between polyps of different hydrozoan species reflect changes in the relative composition of the oral region, body column, and polyp base, and in the placement of structures such as tentacles and gonophores along the axis of the polyp.

The family Hydractiinidae displays a diverse array of polyp forms. A *Podocoryna* polyp has a

hypostome and a single whorl of elongate (filiform) tentacles at the oral end, bears gonophores from its mid-body column, and, at its base, remains attached to the stolon from which it budded (Fig. 1(A)). The oral end of a gastrozooid polyp of *Hydractinia*, a close relative of *Podocoryna*, is very similar to a polyp of *Podocoryna*, yet the rest of *Hydractinia* is markedly different. Polyps at the periphery of a *Hydractinia* colony are attached to stolons (not shown), and at the center of the colony, the polyps' epithelium is continuous with a stolonal mat (Fig. 1(B)). *Hydractinia* does not bud gonophores from its gastrozooid body column; instead there is a division of labor, with gastrozooid polyps strictly for feeding, and gonophores budded from the body column of a gonozooid polyp, which lacks a mouth and the long narrow (filiform) tentacles of the gastrozooid (Fig. 1(B)). *Hydractinia* displays two other polymorphic polyps, the dactylozooid and tentaculozooid (not shown), which also possess distinct morphologies reflecting their different oral/aboral patterning

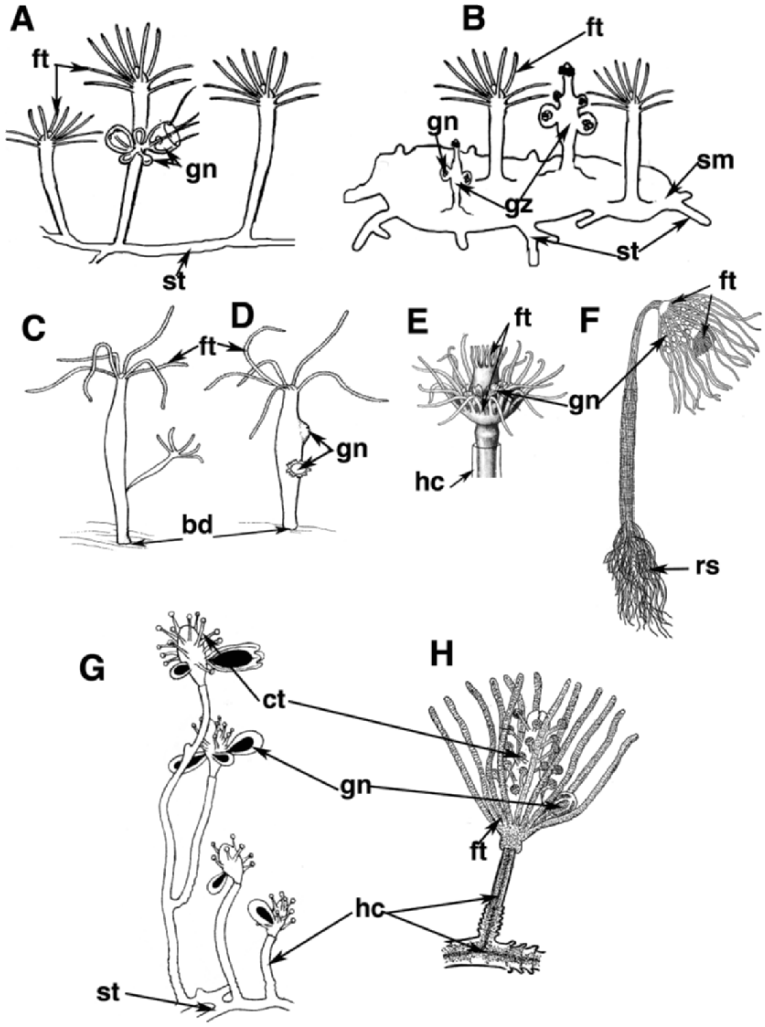

Figure 1. Comparison of different polyp morphologies. (A) *Podocoryna*. (B) *Hydractinia*. (C) *Hydra* with asexual buds. (D) Female *Hydra* with gonophores. (E) Colonial *Tubularia*. (F) Solitary *Tubularia*. (G) *Coryne*. (H) *Pennaria*. gn, gonophores; gz, gonozooid; ft, filiform tentacles; ct, capitate tentacles; st, stolon; sm, stolonal mat; hc, hydrocaulus, bd, basal disk; rs, root structures. (A), modified from Berrill (1961). (B), modified from Berking (1998). (C)–(E), modified from Bayer & Owre (1968). (F) and (H), modified from Hyman (1940). (G), modified from Ruppert & Barnes (1994).

(e.g. Huxley, 1859; Agassiz, 1865; Metschnikoff, 1874; Brooks, 1886; Haeckel, 1888). The distantly related fresh-water solitary *Hydra* has an oral end similar to a *Podocoryna* and *Hydractinia* gastrozooid, with a single row of filiform tentacles surrounding a hypostome with a mouth. *Hydra* reproduces asexually through the formation of buds on the body column (Fig. 1(C)), and during its sexually reproducing phase, gonophores develop along the body column (Fig. 1(D)). Ectodermal cells at the base of the *Hydra* polyp, referred to as the basal disk (Fig. 1(C) and (D)), are specialized morphologically and adhere to the substrate. A polyp of the genus *Tubularia* displays two whorls of filiform tentacles, an oral one surrounding the hypostome and mouth, and an aboral one at the base of the polyp. *Tubularia* buds gonophores on its body column just distal to the aboral tentacles (Fig. 1(E) and (F)). The polyp in colonial species of *Tubularia* is attached to hydrocauli at its base (Fig. 1(E)), whereas that in solitary species possesses, at its base, root-like structures that serve as holdfasts to the substrate (Fig. 1(F)). Tentacles of a polyp of *Coryne* are not in whorls, but are scattered throughout the length of the polyp; moreover, they are short and round (capitate). *Coryne* gonophores bud from the body column, and polyps are attached to short hydrocauli stalks that branch from stolons (Fig. 1(G)). A *Pennaria* polyp possesses scattered capitate tentacles, similar to *Coryne*, as well as an aboral row of filiform tentacles, and polyps are located at the terminal ends of the hydrocauli (Fig. 1(H)).

For a polyp to attain its particular morphology, signaling processes must be acting to specify the appropriate placement of structures along the polyp axis. Changes in the regulation of these signaling processes could result in diverse morphologies and may be an important component underlying the evolution of polyp diversity within the hydrozoa.

Colony morphology and diversity

Hydrozoans not only display a number of different polyp morphologies, but are also noted for their diverse colony morphologies. Figure 2 shows some examples of hydrozoan colony forms. Hydrozoan colonies can be encrusting (Fig. 2(C)), arborescent (Fig. 2(A), (D) and (E)) or pelagic (Fig. 2(B)). They can possess irregularly- (Fig. 2(A) and (C)) or regularly-spaced branches (Fig. 2(B), (D) and (E)), and the branches can be spaced relatively close or far apart. Polyps can be spaced irregularly (Fig. 2(A) and (C)) or regularly (Fig. 2(D) and (E)), and in polymorphic colonies, the polyp types can have a random distribution (not shown) or a precise position within the colony (Fig. 2(B)). Determination of gross colony morphology has been well studied from ecological, phylogenetic, structural, and physiological perspectives (e.g. Buss, 1979; Jackson, 1979; Cheetham et al., 1980; McFadden et al., 1984; Blackstone and Buss, 1991; Dudgeon and Buss, 1996; Blackstone, 1998; Sanchez et al., 2003), but little is known of the molecular mechanisms underlying the patterning of colony form.

Stolons and hydrocauli are epithelial tubes lined with endoderm surrounded with ectoderm that secretes an acellular perisarc. The thickness of the perisarc has been implicated as an important component in determining whether there are upright hydrocauli, which results in an arborescent form, or a creeping hydrohizal network of stolons, which results in an encrusting form (Marfenin & Kosevich, 2004). Developmentally, stolons and hydrocauli both elongate, branch, and bud or differentiate into polyps, and therefore hydrocauli and stolons are treated interchangeably herein.

Colony patterning can be divided into distinct processes as summarized by Braverman (1974). Colony form is determined by (1) rate of extension of stolons, (2) sites of branching, and (3) budding of polyps (Fig. 3). In polymorphic colonies, colony form may be determined by a fourth feature, the positioning of polyp polymorphs. Through these processes, the colony achieves its distinct shape and organization, and therefore the regulation of positional information along a developing stolon axis is an important component of colony patterning.

The role of axial patterning genes in hydrozoan development and evolution

In the past decade, a number of different developmental regulatory genes have been isolated and characterized in *Hydra*, *Hydractinia*, and

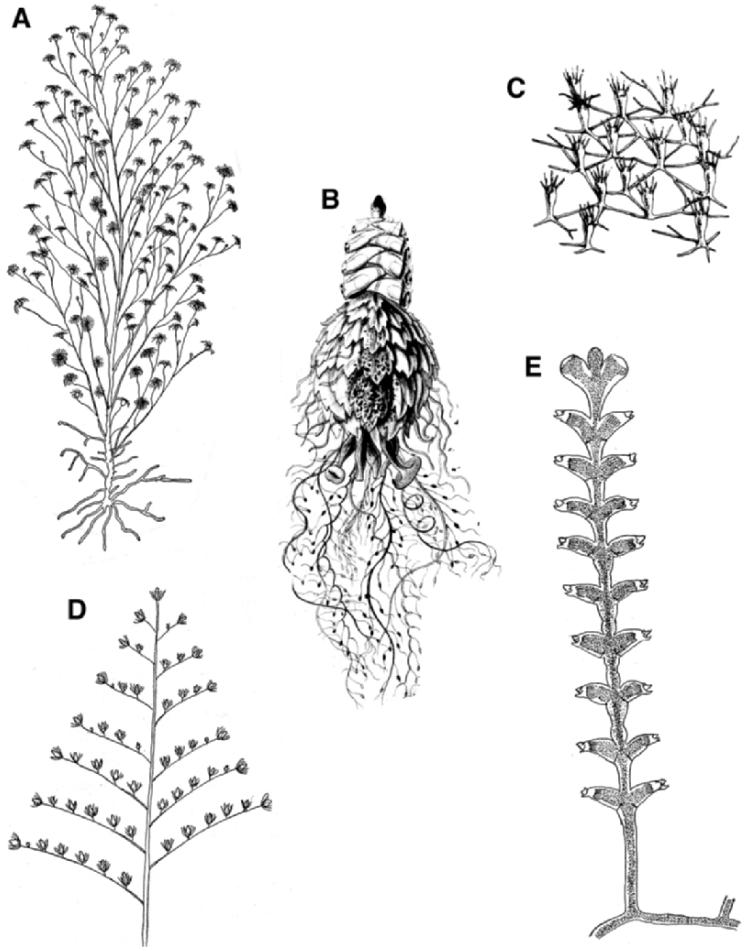

Figure 2. Hydrozoan colony diversity. (A) *Eudendrium*; (B) *Algalma*; (C) *Podocoryna*; (D) *Pennaria*; (E) *Halecium*. (A), (D) and (E) modified from Hyman (1940). (B) modified from Haeckel (1888). (C) modified from Blackstone & Buss (1993).

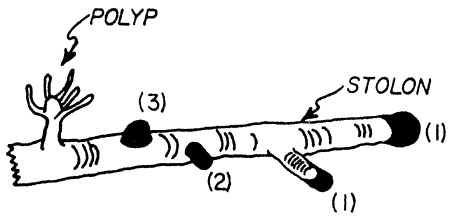

Figure 3. Patterning processes of colonial hydroids. (1) stolon extension; (2) stolon branching; (3) new polyp budding. From Braverman (1974).

Podocoryna. The patterns of expression during normal development and, in some cases, regeneration, have implicated many of these genes as important in axial patterning of the polyp. Table 1 summarizes genes that have been implicated in hydrozoan axial patterning.

These studies provide strong evidence that axial patterning in polyps depends on the proper spatial expression of developmental regulatory genes along the oral/aboral axis. Two studies comparing the expression of developmental regulatory genes in *Hydractinia* polyp polymorphs suggest that changes in the expression of regulatory genes may dictate differences in polyp axial patterning.

The parahox gene Cnox-2 *in the inhibition of oral structures*

Cnox-2 was one of the first developmental regulatory genes isolated in a cnidarian (Schierwater et al., 1991). In *Hydra*, *Cnox-2* is expressed in

Table 1. Summary of genes implicated in axial patterning in hydrozoans

Gene	Position of expression in adult polyp	Proposed axial function	References
HyWnt	Apex of hypostome (Hv)	Organizer	Hobmayer et al. (2000)
CnOtx	Ectoderm of budding site (Hv)	Formation of new axis	Smith et al. (1999)
HyBra1	Endodermal epithelial cells of hypostome (Hv)	Hypostome formation	Technau & Bode (1999)
Cnox-2(4)	Ectodermal apex of hypostome (Pc)	Specification of apical region	Masuda-Nakagawa et al. (2000)
Cn-ems	Endodermal gland cells in hypostome (Hs)	Hypostome specification	Mokady et al. (1998)
prd-a	Nerve cells in hypostome and tentacles (Cv)(Pc)	Apical differentiation	Gauchat el al. 1998
Budhead	Endoderm of lower half of hypostome and tentacles (Hv)	Head maintenance	Martinez et al. (1997)
Cngsc	Endoderm of hypostome/tentacle border (Hv)	Hypostome/tentacle border specification	Broun et al. (1999)
HyAlx	Ectoderm of tentacle/tentacle zone border (Hv) (Hm)	Tentacle specification	Smith et al. (2000)
HvPKC-2	Endoderm of foot and apex of hypostome (Hv)	Head and foot patterning	Hassel et al. (1998)
HTK7	Ectoderm of proximal tentacles and body column/basal disk transition	Tentacle and foot cell differentiation	Steele et al. (1996)
Cnox-2	Ectoderm of body column (Hv) (Hs) and stolonal mat (Hs)	Head inhibitor	Shenk et al. (1993a) Cartwright et al. (1999) Cartwright & Buss (1999)
CnNk-2	Endoderm of peduncle (Hv) (Hm) (Ho)	Specification of basal disk	Grens et al. (1996)
Shin-guard	Ectoderm of peduncle (Hv)	Specification of basal disk	Bridge et al. (2000)
Manacle	Ectoderm of peduncle/basal disk boundary (Hv)	Specification of basal disk	Bridge et al. (2000)

Hv, *Hydra vulgaris*; Hm, *Hydra magnipapillata*; Ho, *Hydra oligactis*; Cv, *Chlorohydra viridissima*; Pc, *Podocoryna carnea*; Hs, *Hydractinia symbiolongicarpus*.

epithelia of the body column and polyp base, with little expression in the oral region (Shenk et al., 1993a). Experimental induction of ectopic oral and aboral structures results in the down regulation and up regulation of *Cnox-2* respectively, suggesting that it plays a role in inhibiting oral structures along the body column (Shenk et al., 1993b). Subsequent studies in *Hydra* have shown that *Cnox-2* binds to a regulatory region of a gene specific to the oral end, *ks1*, suggesting that *Cnox-2* may act to inhibit the expression of *ks1* (Endl et al., 1999).

Comparative evidence in *Hydractinia* polyp polymorphs further supports the idea that the role of *Cnox-2* is to inhibit oral structures. In *Hydractinia* gastrozooids, *Cnox-2* has a similar expression pattern to that of *Hydra*, with a high level of expression in the body column and a low level in the oral region (Fig. 4(A); Cartwright et al., 1999). *Hydractinia* gonozooids lack the oral structures of the gastrozooids (Fig. 1(B)) and possess high, even levels of *Cnox-2* expression throughout the polyp (Fig. 4(B); Cartwright et al., 1999). These results are consistent with *Cnox-2* playing a role in inhibiting oral structures along the body column, and suggest that changes in the regulation of this gene may be, in part, responsible for the differences in oral/aboral patterning in *Hydractinia* polyp polymorphs (Cartwright et al., 1999).

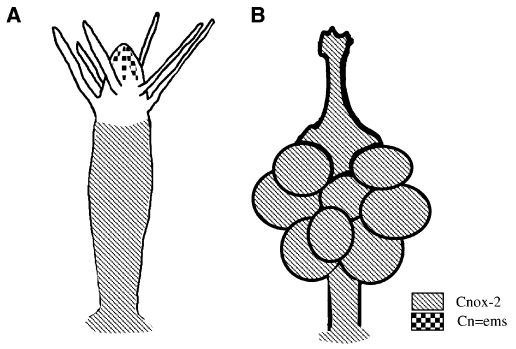

Figure 4. Cnox-2 and *Cn-ems* expression in *Hydractinia*. (A) *Hydractinia* gastrozooid. (B) *Hydractinia* gonozooid.

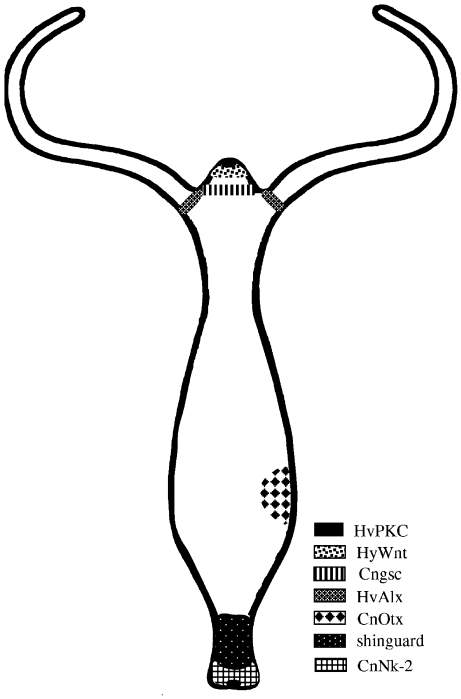

Figure 5. Expression patterns of several axial patterning genes in an adult hydra.

Cn-ems *is specific to gastrozooid structures*

The hypostome of a polyp is lined with endodermally derived longitudinal ridges called taenoilae, which consist of gland cells and epithelial cells (Campbell, 1967; Braverman, 1968). In *Hydractinia*, taenoilae are found only in the hypostomes of gastrozooids, being absent in non-feeding polyps, which lack hypostomes. Mokady et al. (1998) found expression of the gene *Cn-ems* restricted to the endodermal epithelial cells of the taenoilae in *Hydractinia* gastrozooids (Fig. 4(A)). *Cn-ems* was not expressed in gonozooids, which lack taenoilae and a hypostome (Fig. 4(B)), suggesting that *Cn-ems* is involved in specification of gastrozooid-specific oral structures.

The patterns of expression of *Cnox-2* and *Cn-ems* in *Hydractinia* provide suggestive evidence that changes in the regulation of these genes could be, in part, responsible for the evolution of polymorphic polyps. No studies, however, have compared expression patterns of regulatory genes among hydrozoan species with different polyp or colony morphologies. In the absence of direct comparative information, hypotheses can be generated from the studies summarized in Table 1 about the role of these genes in hydrozoan evolution. Some such hypotheses are presented below and await testing through comparative investigation of these genes.

Cngsc *and* HyAlx *in the development and evolution of tentacle placement*

In *Hydra*, the gene *Cngsc* is expressed in a ring of endodermal cells at the hypostome/tentacle zone border, suggesting that *Cngsc* may play a role in defining the region where tentacles bud (Fig. 5) (Broun et al., 1999). Expression of *Cngsc*, in species whose tentacle organization differs from that of *Hydra*, is predicted to coincide with the region(s) of tentacle placement. For example, *Cngsc* is predicted to be expressed in two discrete zones in *Tubularia*, at the oral region defining the oral whorl of tentacles and at the base of the polyp defining the aboral whorl. In species with tentacles scattered throughout the polyp, such as in *Coryne*, *Cngsc* is predicted to have a broader zone of expression in the region of the scattered tentacles. *Pennaria* is predicted to possess a discrete ring of *Cngsc* expression at the base of the polyp, defining the aboral tentacle whorl, and a broader zone of expression distally, defining the scattered tentacle region.

The ring of *Cngsc* expression defines the region along the aboral/oral axis where tentacles bud, but does not define the radial arrangement of tentacles within the zone. The gene *HyAlx* has been implicated in specification of tentacle tissue in the region from which tentacles bud. In *Hydra*, *HyAlx* is expressed in a band of ectodermal epithelial cells at the tentacle/tentacle zone border, defining rings of tissue where tentacles emerge from the body column (Fig. 5; Smith et al., 2000). As cells move from the body column into the tentacles, they are specified at the margin to form tentacle tissue. The expression pattern of *HyAlx* suggests that it is involved in tentacle tissue specification (Smith et al., 2000). If *HyAlx* functions within the zone defined by *Cngsc* to specify tentacles, it is predicted to be expressed at the region where tentacles bud, determining whether tentacles are arranged in a whorl, as in *Tubularia* (Fig. 1(E)), or scattered along the body column, as in *Coryne* (Fig. 1(H)). A better understanding of the regulation of *HyAlx*

and yet uncharacterized tentacle specification genes would greatly enhance understanding of the mechanisms behind tentacle placement and organization in various hydrozoan species.

HvPKC2 *in the differentiation of terminal ends and in the evolution of solitary forms*

In *Hydra*, the gene *HvPKC-2* has been implicated in the specification of both oral and aboral ends of the polyp, being expressed in endodermal epithelial cells in two regions, at the tip of the hypostome and in the basal disk (Fig. 5; Hassel et al., 1998). *HvPKC-2* is a member of the protein kinase C family that plays important roles in cellular signaling. This suggests that *HvPKC-2* may be involved in signaling cells that have reached a terminal end (either oral or aboral) of the polyp. Characterization of the expression pattern of *HvPKC-2* in colonial hydrozoans would provide insight into its function, and be key to testing evolutionary hypotheses. Lack of expression at the aboral end of a colonial polyp that is attached to a stolon and lacks an aboral terminal end would provide additional evidence that *HvPKC-2* is involved in terminal specification, and that the up regulation of terminal differentiation genes such as *HvPKC-2* in the aboral end may play an important role in the evolution of solitary forms from colonial relatives.

CnNK-2 *and* shinguard *in basal disk specification and its possible role in colonial species*

The basal disk is a specialized structure specific to *Hydra* that functions in attaching the polyp to the substrate. Many developmental regulatory genes have been implicated in patterning the aboral end, specifically in specification of basal disk tissue. The genes *CnNK-2* and *shinguard* are expressed in the endoderm and ectoderm, respectively, in the region of the body column just distal to the basal disk (Fig. 5), and are implicated in specification of basal disk tissue as the cells pass through the body column/basal disk border (Grens et al., 1996; Bridge et al., 2000). It is unclear what role these genes would have in other hydrozoans. If they have a more general role in specifying the aboral end of the polyp, they would be predicted to be expressed in the aboral end of other hydrozoan species. Alternatively, if *CnNK-2* and *shinguard's* function in basal disk specification evolved in the hydra lineage, their function in aboral patterning would be specific to *Hydra*. Comparative investigations of these genes in other hydrozoans would shed light on this issue.

HyOtx *in* Hydra *budding and its potential role in stolon patterning*

Most developmental regulatory genes characterized in *Hydra* are expressed in similar patterns in polyp buds and adults, suggesting dual roles in setting up and maintaining axial patterning. The gene *HyOtx* is an exception to this generalization. In *Hydra*, its expression is restricted to ectodermal cells of the adult body column in the region that is ultimately displaced into a bud (Fig. 5), which suggests that *HyOtx* plays a role in directing cellular movements of the body column as it evaginates (Smith et al., 1999). Colonial hydrozoans do not bud polyps from the column but other processes such as gonophore budding, stolon branching, and polyp budding from stolons require evagination and cellular rearrangements (Hyman, 1940), so any or all of these processes may involve *HyOtx*.

Wnt *and the hydrozoan organizer*

The apex of the hypostome in *Hydra* is important in maintaining axial patterning of the polyp. This region has been referred to as the '*Hydra* organizer' because, in transplantation experiments, it has the capacity to induce host tissue to form a second axis (Broun & Bode, 2002). The molecular basis of the organizer is largely unknown, but recent evidence has implicated the Wnt pathway. In *Hydra*, the *Wnt* homolog, *HyWnt*, is expressed in the apex of the hypostome in the region corresponding to the *Hydra* organizer (Hobmayer et al., 2000). Treatment of *Hydra* with 2 mM LiCl and diacylglycerol results in development of ectopic oral structures along the body column (Muller, 1989; Hassel et al., 1993). These agents indirectly mimic the action of *Wnt* (e.g., Cook et al., 1996; Chen et al., 2000; Phiel & Klein, 2001), providing further evidence that the *Wnt* pathway is involved in setting up and in maintaining the *Hydra* axis. In colonial hydrozoans, polyp axes are induced along

the developing stolons from which they bud. Evidence in *Hydra* suggests that the *Wnt* pathway may be acting to induce polyp budding along stolons. If this is the case, *Wnt* is predicted be expressed in colonial hydrozoans in the regions of stolons where polyps bud.

Conclusions

Hydrozoans exhibit a wide range of diversity in polyp and colony morphologies, much of which can be attributed to differences in polyp and stolon axial patterning. In recent years, many developmental regulatory genes have been implicated in the axial patterning of polyps. Changes in the structures/regions that these genes have been implicated to specify have been a prominent component in hydrozoan evolution and therefore evolutionary changes in the regulation of these genes may be an important mechanism underlying hydrozoan diversity. As more comparative information becomes available, our understanding of the role of these genes in hydrozoan evolution will be greatly enhanced. Comparative investigations may reveal that changes in regulation of positional information along the axes of polyps and stolons may be, in large part, responsible for the remarkable diversity of colonial forms.

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Morphogenetic evolution of hydroid colony pattern

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Key words: hydroid colony, heterochrony, colony integration, Hydrozoa

Abstract

A scheme of evolution of hydrozoan colony pattern is proposed based upon the consideration of macromorphogenesis. Four main processes play decisive roles: (1) hard skeleton formation by soft tissues, (2) changes in duration of the growth phase relative to the transition to differentiation in interdependent zones of growth, (3) ratio in growth rates between adjacent zones of growth within the rudiment, the shoot, or the whole colony, and (4) spatial relationships among growth zones. The main tendency in morphological evolution of the hydroids is an increasing integration of the colony as revealed by increasing complexity of its structure. That is from a temporary colony towards the permanent one with highly organised shoots, as hydranths and branches are localised in a strictly arranged manner. An analysis of diverse data allows one to state that the main morphogenetic mechanism of increasing complexity in the hydroid colony is convergence, then fusion, of adjacent growth zones, a variant of heterochrony.

Introduction

Most invertebrates belonging to the phylum Cnidaria form colonies, possessing what is now called a modular level of organisation (Chapman, 1981; Jackson & Coates, 1986). Despite there being diverse morphological features among different groups, the structure and organisation of colonies often look very similar. It is now obvious that gross morphology of the colony evolves in parallel in different groups independent from their phylogenetic relations (Sánchez, 2004).

This paper considers the main factors in the morphogenetic evolution of colony pattern using the polyp stage of hydroids, most of which are modular, as the model. By 'morphogenetic evolution' we mean the set of regulatory processes that leads to increasing regularity and complexity of hydroid colony structure, primarily of the shoots. Here we shall focus mainly on the subclass Leptolida (the old terminology), with examples from several families. But the processes discussed are not specific to these groups, for they are revealed also in many taxonomic groups of hydroids and in other modular organisms. While this study will not constitute a total and complete analysis of the morphogenetic evolution of hydroid colonies, the main stages are considered – those that define the major directions in morphological evolution within the groups of high taxonomic rank.

Our focus will be on a consideration of macromorphogenesis, or morphogenesis at the supracellular level, rather than on the molecular and biochemical mechanisms that underlie these processes. Morphogenesis surely depends upon gene expression, but little is known how it is achieved. Moreover, the control of morphogenesis cannot be reduced only to genetic regulation. The greater if not decisive role is played by the rules and laws of the theory of complex systems. Therefore, the interaction of elements within the whole and the influence of the environment upon them undoubtedly play a role in the realisation of

morphogenesis. This means that morphogenesis bears no direct relationship to gene expression.

The main parameters considered are differences (interdependence) in growth rates of different rudiments in the colony, growth of certain rudiments in different directions, the localisation of growth and branching zones, shape of the structure, skeleton formation, etc. Using such morphogenetic characters, nearly 100 years ago Kühn (1914) proposed a simple classification of growth types in colonial hydroids, which was further modified by Naumov (1960). Surprisingly, this approach was not developed further and was not applied in full measure. Perhaps it is the difficulty of investigating growth and morphogenesis in colonial hydroids that led to a distrust of speculative models for them. However, the morphogenetic approach is insufficiently developed even with regard to a description of diversity in plant forms, which are more accessible.

In this paper we took into account the series of papers by Berrill (1949a, b, c, 1950), and 'the theory of biological (embryonic) field' by Gurwitsch (1922) and his followers (Beloussov & Ostromova, 1969; Beloussov et al., 1972; Beloussov & Dorfman, 1974), including the only papers of their kind by Beloussov (1975, 1991) on the 'parametric system of hydroids'. However, such works are still few in number, so the method of morphogenetic analysis in classification of forms remains poorly developed.

Materials and methods

The data and theoretical ideas set forth in this work are based on detailed investigation of all aspects of the biology, growth, morphogenesis, and ecology in 10 species of colonial hydroids: athecates *Clava multicornis* (Forskal, 1775), *Cordylophora inkermanica* (Marfenin, 1983), and *Tubularia larynx* (Ellis & Solander, 1786), and thecates *Dynamena pumila* (L., 1758), *Gonothyraea loveni* (Allman, 1859), *Laomedea flexuosa* (Hincks, 1861), *Moerisia maeotica* (Ostroumow, 1896), *Obelia geniculata* (L., 1758), *Obelia longissima* (Pallas, 1766), and *Orthopyxis integra* (McGillivray, 1842). The results were presented in a series of papers (Marfenin & Burykin, 1979; Burykin & Marfenin, 1983; Kosevich & Marfenin, 1984; Marfenin, 1984a, 1985a, b, 1987, 1999; Marfenin & Kosevich, 1984; Marfenin & Homenko, 1988; Leontovich & Marfenin, 1990) and in two monographs (Marfenin, 1993a, b).

Nine species were studied in less detail: athecates *Sarsia tubulosa* (M. Sars, 1835), *Sarsia producta* (Wright, 1858), *Rhizogeton nudum* (Broch, 1903), and *Bougainvillia* sp. and thecates *Abietinaria abietina* (L., 1758), *Diphasia fallax* (Johnson, 1847), *Hydrallmania falcata* (L., 1758), *Sertularella gigantea* (Mereschkowsky, 1878), and *Sertularia mirabilis* (Verrill, 1873).

The method of colony mapping by Marfenin (1980) was applied to investigations of living material. It enables one to fix all spatial changes in form and size of the colony. Time-lapse filming and video recording were also used extensively. To supplement these techniques, morphometry of colony elements and histological surveys were also done. Ecological studies were performed under laboratory conditions (Malutin & Marfenin, 1988; Marfenin & Malutin, 1994; Orlov & Marfenin, 1995a, b) as well as in the natural environment (Belorustseva & Marfenin, 2002).

Results

Four processes collectively play decisive roles in providing the evolutionary growth of complexity of hydroid colony patterns. These are (1) formation of a hard skeleton, perisarc, by soft tissues, (2) changes in ratio between duration of the growth phase and transition to differentiation in interdependent zones of growth, (3) differences in growth rates between adjacent zones of growth within the rudiment, the shoot, or the whole colony, and (4) changes in spatial relationship of growth zones.

Figure 1 shows a simplified scheme of the evolution of colony structure in the Hydrozoa. It illustrates the result of the manifestation of the main morphogenetic tendency: from temporary colonies – through stolonal colonies of different types – to several main trends of increasing complexity in the development of branching shoots. One can see that this scheme corresponds to the subdivision of hydroids into its main morphological groups: the athecates, thecates, hydrocorals, siphonophores, etc. For simplicity, representatives of only certain hydroid families are shown, and

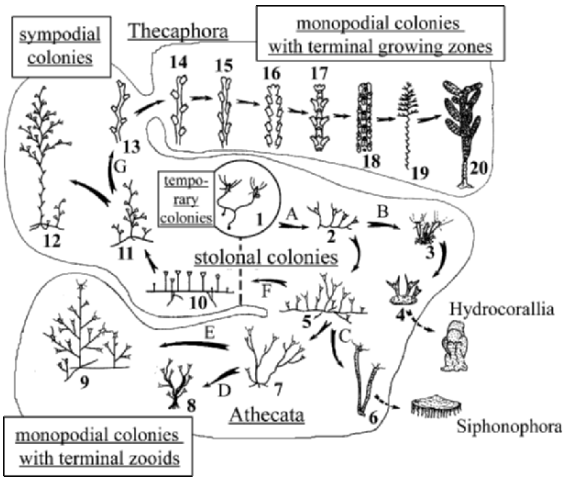

Figure 1. Proposed scheme of morphogenetic evolution of colony pattern in Hydrozoa. Numbers correspond to the species used as examples. *Limnomedusae*: (1) *Moerisia maeotica* (Ostroumow, 1896), (2) *Moerisia pallasi* (Derzhavin, 1912). *Athecata* (*Anthomedusae*): (3) *Clava multicornis* (Forskal, 1775), (4) *Hydractinia echinata* (Fleming, 1828), (5) *Cladonema radiatum* (Dujardin, 1843), (6) *Tubularia* sp., (7) *Coryne lovenii* (M. Sars, 1846), (8) *Eudendrium rameum* (Pallas, 1766), (9) *Cordylophora caspia* (Pallas, 1771). *Thecaphora* (*Leptomedusae*): (10) *Orthopyxis integra* (McGilliwray, 1842), (11) *Gonothyraea loveni* (Allman, 1859), (12) *Obelia longissima* (Pallas, 1766), (13) *Sertularella pellucida* (Jaderholm, 1908), (14) *Sertularia nana* (Hartlaub, 1901), (15) *Sertularia suensoni* (Levinsen, 1913), (16) *Thuiaria carica* (Levinsen, 1983), (17) *Sertularia staurotheca* (Naumov, 1960), (18) *Thuiaria mereschkowskii* (Kudelin, 1914), (19) *Thuiaria tetrastriata* (Naumov, 1960), (20) *Thuiaria zachsi* (Feniuk, 1947). Main points of changes in colony structure: (A) from temporary to permanent colony; (B) stolonal colonies become more compact, formation of stolonal mat with thickened perisarc; (C) increasing of hydranth size and reduction of stolonal structures; (D) formation of polysiphonal shoots; (E) regularity in branching of shoots; (F) from athecates to thecates; (G) decreased hydranth size, increased branching regularity and general complexity of colonies. Dotted line (between 5 and 10), border between athecates and thecates.

among them Sertulariidae looks mostly indicative, concerning evolution of shoot's construction.

Formation and functions of the outer skeleton

If we accept that colonial hydrozoans have originated from solitary ones on the bases of unfinished vegetative reproduction (Spenser, 1898; Ivanov, 1968; Beklemishev, 1969), then perisarc could help keep the zooids connected with one another. First it provided protection for dormant stages of the organism. Later the perisarc came to be used for

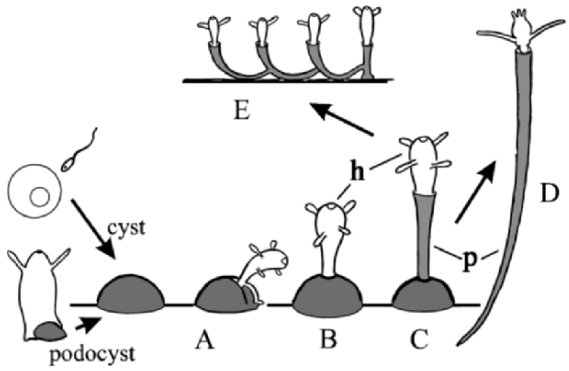

Figure 2. Acquisition of perisarc and alteration of its function. (A) Protection of dormant stages. (B) Attachment to substratum. (C) Protection plus support. (D) Support of elevated structures. (E) Connection of zooids. h Hydranth (zooid); p, pedicel of a hydranth. Perisarc is shaded grey. Podocyst: dormant stage, also used for asexual reproduction in some species of hydroids, more common for scyphozoans; a type of cyst formed by the basal part of a solitary polyp (foot) and covered with perisarc.

firm attachment of the organism to its substrate. As the transition occurred from temporary to permanent colonies, it appeared to be effective both in maintaining the inter-zooid connection and in providing support and protection of those structures elevated off the substrate (Fig. 2). But, by its rigidity, it imposes restrictions on the shape and size of the enclosed soft tissues by limiting further growth in certain directions. On the whole, these supportive and protective functions provide the basis for evolutionary development of elevated structures – hydranths with long pedicels or shoots with numerous hydranths.

Changes in duration of growth of the rudiment before and after differentiation of a hydranth

The evolutionary development of shoots bearing numerous hydranths went along two main alternative paths (Fig. 3):

(1) Athecate hydroids are characterised by an early development of hydranth structures – tentacles and hypostome – on the rudiment, followed by further growth of this poorly differentiated hydranth for some period of time. In addition, most athecates prolong functioning of the growth zone beneath the terminal hydranth, thereby causing formation and elongation of its pedicel and giving rise to lateral branches.

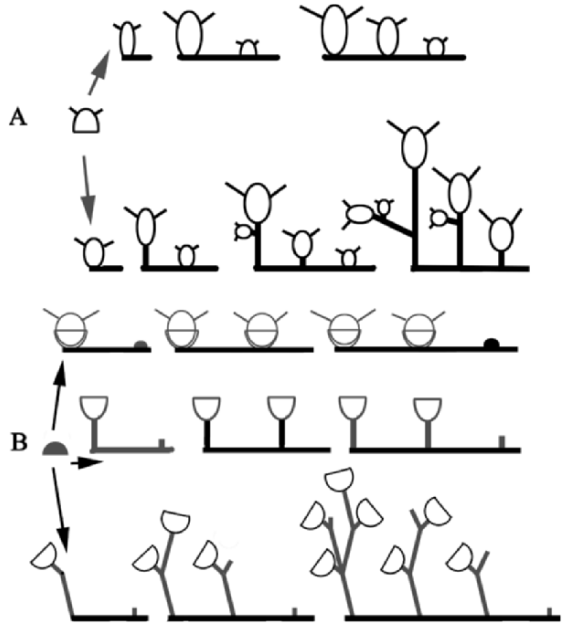

Figure 3. Two strategies in shoot evolution. (A) Long-functioning sub-terminal growth zone (athecates). Continuous branch elongation and hydranth growth. Top series e.g., *Clava multicornis*, *Rhyzogeton nudum*, *Hydractinia* spp. Bottom series *Cordylophora caspia*, *Bougainvillia* spp., *Pennaria* spp. (B) Temporal terminal growth zone (thecates). Fixed sizes of the hydranths and internodes. In middle and bottom series only hydrothecae without hydranths are shown. Top series – e.g., *Filellum serpens*, *Cuspidella mertensii*. Middle series – e.g., *Orthopyxis* integra, Calicella syringa. Bottom series – e.g., *Obelia* spp., *Campanulina lacerate*. In all cases, distances between hydranths or shoots remain fixed after their formation.

Growth of the main and lateral pedicels continues indefinitely.

(2) Thecate hydroids display another way. Limited duration of growth zone function produces a full-sized hydranth incapable of further growth. If a pedicel forms beneath it, its elongation is of limited duration, so that if the pedicel is to continue to lengthen, successive emergence of new growth zones is necessary.

Arrangement of growth zones within the colony

In the monopodial shoots of athecates, each branch has one growth zone. Due to the continuous nature of growth in this zone, the distance between hydranths increases through time (Fig. 3A, lower scheme). Such structural organisation from the morphogenetic point of view is a blind alley.

By contrast, in the sympodial shoots of thecates, the growth zone at the tip of the pedicel functions only temporarily, until a hydranth differentiates at its tip (Fig. 4). Then a new growth zone must appear to continue elongation of the shoot. That is, growth of the shoot is cyclic. In such cases, further evolution of the thecate shoot structure becomes possible due to changes in timing between the completion of hydranth formation (the cessation of activity in one growth zone) and emergence of the next growth zone. The shorter this time interval, the less is the distance between the two adjacent rudiments (Fig. 5A). Figure 5B illustrates the morphological consequences, using several species of the Campanulariidae. Note that when the time interval is short enough, the two growth zones are so close that they fuse. When this occurs, as in some species of *Halecium*, the growing tip divides into two rudiments, one giving rise to a hydranth, the other to the start of the next shoot internode.

From the *Halecium* pattern of sympodial growth, little further change is required to transition to the monopodial form of shoot growth with a terminal growth zone. Indeed, this transition is evident when comparing several genera of the Sertulariidae (Fig. 6). The transitions from I to IV in Figure 6 illustrate an increasing physical association between the hydrotheca and the perisarc of the next internode rudiment, and this is determined by how much the hydrotheca grows after splitting of the tip. If the hydranth rudiment grows for some time after the tip splits, its hydrotheca will be joined to the stem only or largely at its base

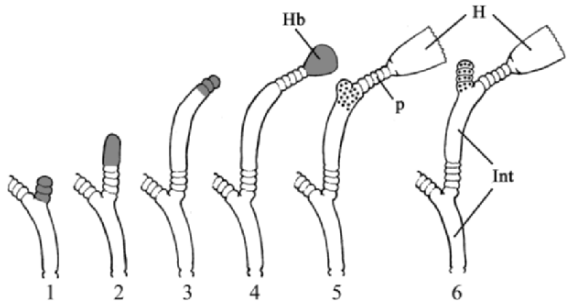

Figure 4. Morphogenetic cycle of the shoot with sympodial mode of growth. 1–6, successive stages. H, hydrotheca; Hb, hydranth bud; Int, shoot internode; p, hydranth pedicel. Consecutive growth zones are shaded grey and dotted respectively.

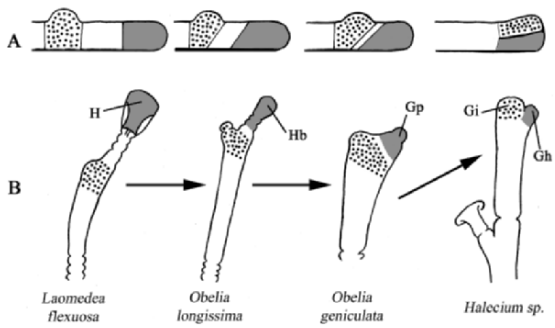

Figure 5. Convergence and fusion of successive growth zones. The terminal growth zone is shaded grey. The next growth zone is dotted. (A) Four steps in the sequence of events, shown schematically, left to right. (B) Examples of each step from three species of Campanulariidae and Haleciidae. Gh, growing zone (rudiment) giving rise to a hydranth; Gi, growing zone (rudiment) giving rise to the next shoot internode; Gp, growing zone started formation of the hydranth pedicel; H, immature hydranth; Hb, hydranth bud.

(Fig. 6 I and II). But if the hydranth completes its growth quickly relative to the rate of tip elongation, its hydrotheca will be fused laterally with the perisarc of the shoot and its orifice will be located at the level of the stem surface (Fig. 6 III and IV). Naumov (1960) was the first who noted the phenomenon of 'hydranth sinking into the hydrophyton,' along with the evolutionary growth of complexity of the shoot structure.

In stolonal colonies, in sympodial shoots, and in monopodial shoots with terminal hydranths, the growing tip represents a radially symmetrical and balanced structure. But the transition toward subdivision of the growth zone into two rudiments opens a new phase in the evolution of shoot morphogenesis and structure. As subdivision of the growing tip into separate hydranth and stem rudiments occurs earlier and earlier prior to differentiation of the hydranth (that is, toward the right side of Figs 5 and 6), the tip becomes asymmetric and unbalanced. The consequences of such a phenomenon are discussed in detail in the works by Beloussov (1975, 1991). The asymmetry of the growing tip underlies the origin of diverse morphologies of different species.

Early emergence of the next shoot internode growth rudiment within the growth zone of the underdeveloped previous internode leads to formation of a shoot bearing hydranths arranged in one, two, or several rows. The number depends upon the number of partitions set up by the growing tip, their mutual spatial arrangement, and their growth rates. On the whole, the morphogenetic cycle of internode formation becomes more complex, including the development not of one but of several hydranths prior to return of the tip's shape back to its starting condition. This can be illustrated by shoot growth in *Abietinaria abietina* (Fig. 7). After separation of the branch and hydranth rudiments in Figure 7A, the growing tip changes significantly in form, becoming less broad. Only after formation of the third hydranth rudiment farther on does the growing tip start to return to its original appearance (compare in Fig. 7, A with G). In this way, the morphogenetic cycle of the shoot internode incorporates the formation of a lateral branch rudiment that leads to regularity of branching (the branches are formed obligatorily in a certain order, at a certain (predictable) position and at certain (and constant) distances from one another).

Ratio between growth rates throughout the colony

The shape of the colony depends in large part upon the relative duration and rate of growth of its parts. This can be illustrated most clearly in the shape of its shoots.

If the branches arise regularly and all grow with equal and constant rates, then the shoot becomes spear-shaped, as in young shoots of *Sertularia murabilis* (Fig. 8A). If the growth rate of the branches slows with age, then the shoot appears lancet-like, as in *Diphasia fallax* (Fig. 8B). If the elder branches resorb, the shoot looks like a pine tree with a naked proximal part, as in old shoots of S. *mirabilis* (Fig. 8C). If the branching and growth rates of the branches are irregular, such shoots attain diverse shapes, as in *Gonothyraea loveni* and *Dynamena pumila* (Fig. 8D). In some species all lateral branches give rise to frustules, the result being that even though many branches are formed, the stem appears to be only weakly branched, as in *Obelia geniculata* (Fig. 8E). Finally, many species from different groups show a definite ratio in the duration and growth rate of branches of successive orders, which leads to a species-specific shape of the shoots, as in *O. longissima* and many species of Aglaopheniidae and Plumulariidae (Fig. 8F). The shape of

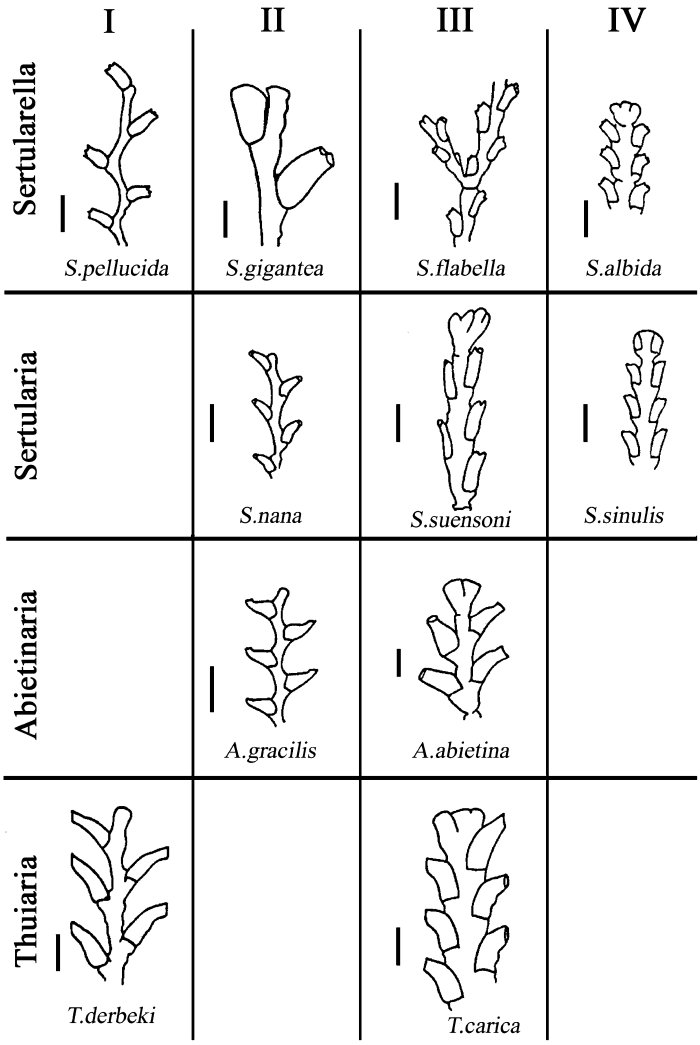

Figure 6. Examples of transition from sympodial mode of shoot growth (I) towards the monopodial with terminal growth zone (IV) within four genera belonging to Sertulariidae. Species names are according to Naumov (1960). Scale bar: top row, 1 mm; the rest, 0.5 mm.

the shoots is achieved due to the differences in the size (length and diameter) of the internodes and duration of growth between the stem and branches of different orders: the higher the branch order, the shorter is the length of the branch. Resorption of the old branches plays a role, too.

Discussion

The main tendency in the evolution of colonial hydroid morphology is integration of the colony gradually toward the level of a unitary, non-modular, organism. In the course of such evolution, the colony as a whole gains individuality as the component zooids loose their individuality (Beklemishev, 1969). With regard to the four morphogenetic processes described above that determine colony form in hydroids, one can imagine an evolutionary tendency toward more complex colony structure: from a temporary colony toward a permanent one with highly organised shoots along which hydranths and branches are localised in a strictly arranged manner.

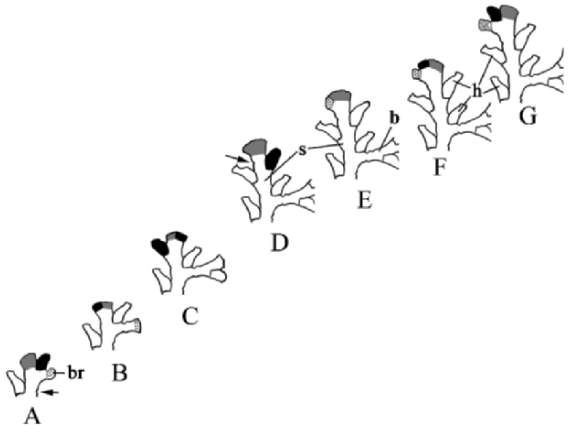

Figure 7. Complex morphogenetic cycle of the shoot internode development in *Abietinaria abietina* (Sertulariidae), including formation of a lateral branch and three hydranths. b, lateral branch; br, branch rudiment; h, hydranth; s, shoot stem. Hydranth rudiment is black, lateral branch rudiment is dotted, stem rudiment is shaded grey. Arrows indicate the furrow on the perisarc surface which is usually believed to be the sign of the borders between the internodes. A–G, successive stages: (A) Beginning of the internode formation; wide growing tip subdivided into tree rudiments. (B) Splitting of the second hydranth rudiment. (C) Splitting of the third hydranth rudiment; end of the internode formation. (D) Beginning of the next internode formation; widening of the growing tip. (E) Splitting of the branch rudiment. (F) Splitting of the first hydranth rudiment. (G) Stage in the stem growth equivalent to A.

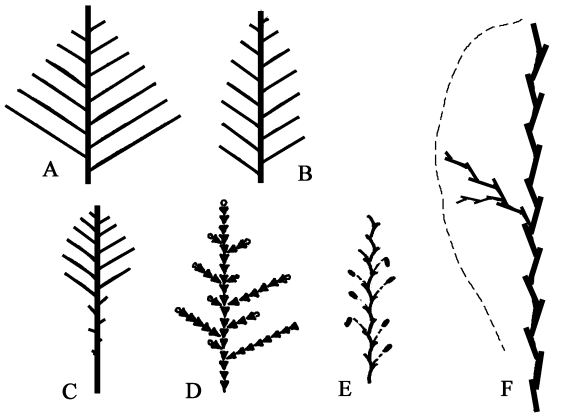

Figure 8. Effect of correlation between growth rates and duration of growth of branches upon the shoot shape in colonial hydroids. (A) Regular branching and constant growth with constant rate. (B) Regular branching and slowing-down of growth rates with age. (C) Like in A or B plus resorption of old branches. (D) Irregular branching and unsteady growth rates. (E) Transformation of lateral branches into frustules. (F) Regular differences in the growth rates and duration of growth between branches of successive orders. For simplicity, only one branch of the first order and one branch of the second order are shown. The contour of the shoot (one side) is outlined by the dotted line; at the base of the stem old branches undergo resorption. In A, B, C, and F, the diameter of the branches is about the same as that of the main stem.

A comparative morphological analysis of a large number of Leptolida species, together with an investigation of shoot morphogenesis in certain species, allow us to assert that the main morphological mechanism for achieving structural complexity in hydroid colonies is the early emergence of the next rudiment or growth zone; that is, a convergence followed by fusion of adjacent growth zones. This is a variant on typical heterochrony, which is well known to play an important role in the evolution of many organisms (Gould, 1977; Raff & Kaufmann, 1983; McKinney & McNamara, 1991). The efficiency of this process is increased by the establishment of a shoot morphogenetic cycle during which branching regularity and hydranth development, its duration, size and shape, are determined.

Convergence of the growth zones (that is, shortening of the period prior to emergence of the next growth zone) during the intermediate stage of increasing of morphogenesis complexity opens up possibilities for variability. Even Berrill (1949a) gave examples of the next terminal hydranth bud emerging at different times in *Obelia geniculata* (Campanulariidae) and *Schizotricha tenella* (Plumulariidae). As a result, growth of the lateral rudiment could even surpass that of the central one that gave rise to it. The evolution of morphogenesis within the Plumulariidae was not studied more, so this example from Berrill adds significant confirmation of the commonness of the patterns under discussion.

One can recognise similar processes in the hydroid group Stylasteridae, in which evolution is affected by calcification of the skeleton (Cairns, 1987). The development of more and more integrated cyclosystems, or a 'cormidium' in general form (Beklemischev, 1969), takes place due to the convergence of local growth zones. Higher morphological integration of the siphonophoran cormus is predetermined by the primordial closing of three growth zones in the larva.

Growth zone integration in the Hexacorallia is achieved in two ways (Marfenin, 1984b). In one, intra-calicle fission takes place earlier and earlier, and as a result the colony is transformed from a

dichotomous one (family Cariophyllidae) to a honeycomb (family Faviidae) and further on into a brain-shape (e.g. genus *Platygyra*). Along the same direction plus predominant lateral branching, increasing structural complexity is realised through the convergence of the growth zones and increasing complexity of the growing tip, as illustrated by the genus *Acropora*.

A decrease in the spatial and temporal interval between the emergence of consecutive rudiments, together with the development of integral (complex, joint) growth zones is a prevailing 'trick' in the evolution of morphogenesis. It can be demonstrated in all modular organisms: different invertebrates, plants, and fungi. These processes can be more easily investigated using colonial hydroids than in other colonial invertebrates, and especially more easily than in unitary organisms.

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Part VI
Reproduction, Development, Life Cycles

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Budding, bud morphogenesis, and regeneration in *Carybdea marsupialis* Linnaeus, 1758 (Cnidaria: Cubozoa)

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Key words: Cnidaria, Cubozoa, *Carybdea marsupialis*, budding, regeneration

Abstract

The cubopolyp of the box jelly *Carybdea marsupialis* (L.) reproduces asexually by lateral budding, and by almost total transformation of the polyp into a cubomedusa. Buds, one or more at a time, emerge laterally from the body column and separate from the parent as secondary polyps bearing hypostome and some tentacle anlagen. We describe at the light microscopic level the consecutive steps of bud formation and propose to discriminate a series of eight developmental stages. Once detached, these buds elongate and pass through a vagile phase, creeping over the substratum with the tentacular region ahead. The vagile time period appears to depend on the culture condition; it ends with the firm attachment of the young polyp which then develops the full complement of tentacles along with further growth. The budding rate is positively correlated with the frequency of feeding, in contrast to conditions leading to medusa formation, and is enhanced in the dark. The 'creeping polyp stage' reveals a high regeneration potential: both oral and aboral fragments, obtained by midbody transection, regenerate the ablated portion within 72 h. Regeneration in fragments excised from the body column restores also polyp morphology according to the original polar organization. Results of pilot experiments involving excision of the tentacle and hypostomial portion of adult budding and non-budding cubopolyps lead us to discuss a possible interplay between regeneration of oral structures and bud formation.

Introduction

Asexual reproduction of the polyp stage by budding off motile propagules which eventually settle and develop into secondary polyps is a common feature in several cnidarian taxa. Many species within the Limnohydrina (e.g. *Gonionemus* spp., *Craspedacusta sowerbyi*) produce vermiform, non-ciliated, creeping frustules which develop polyp features only after attachment to the substratum (Tardent, 1978; Werner, 1984, for review). A particular type of larva-like, ciliated propagule emerging from the lower part of the polyp's calyx is restricted to species of the scyphozoan order Rhizostomeae (Hofmann & Crow, 2002, for review). Life-cycle studies in species of the cubozoan genera *Tripedalia*, *Carybdea* and *Chironex* (e.g. Werner et al., 1971; Cutress & Studebaker, 1973; Yamaguchi & Hartwick, 1980) have briefly shown that cubopolyps, apart from total or almost total transformation into cubomedusae, reproduce asexually by lateral budding of propagules which, after detachment from the parent, develop into secondary polyps. These propagules share the creeping mode of locomotion with the frustules of the Limnohydrina, but with reversed polarity. They develop, however, like buds in *Hydra* spp., already a hypostome and some tentacles at the

time of detachment, and are able to feed. They were termed the 'creeping polyp stage.'

The present account aims at providing a more detailed description of the budding process in *Carybdea marsupialis* Linnaeus 1758 at the light-microscopical level, thereby proposing to discriminate eight successive stages. It further provides, for the first time, data on budding rates under different feeding and light regimes, complementing the staging and experimental induction of medusa formation in this species published on by Stangl et al. (2002). The study mentions briefly the further development of the bud, and describes results of a set of transection and regeneration experiments performed on both adult, budding, and non-budding polyps, and on the creeping polyp stage.

Material and methods

Stock cultures of *Carybdea marsupialis* polyps (kindly provided by R. Golz) were maintained in glass and styrene dishes filled with 200 (or 500 ml respectively) of pasteurized natural sea water from the North Sea, and propagated asexually in the laboratory at 23 °C. The cubopolyps were fed *Artemia salina* nauplii twice a week. Containers were cleaned and the sea water replaced about 4 h after feeding. The light regime was adjusted to long-day conditions (16 h light: 8 h dark). For some experiments (see below) cultures were protected from light by dark covers, and/or fed more frequently. Transection experiments on adult polyps or on detached buds were performed using sharpened tungsten needles on individuals collected from the cultures, washed three times in antibiotic-containing seawater (ABS, as detailed below), and immobilized in Ca^{2+}-free artificial, antibiotic-containing seawater for 20–30 min. Fragments were placed individually in wells of 24-well tissue culture plates each in 1 ml of ABS made up with 100 mg penicillin, 100 mg streptomycin, and 130 mg neomycin per liter pasteurized sea water. Development was scored under a dissecting microscope daily, sometimes at shorter intervals, for 2–7 days. Microphotographs were taken using either an Axiophot (Zeiss) microscope, an Olympus IMT2 inverted microscope, or an Olympus OM2n camera mounted on an Olympus dissecting microscope, on Kodak professional color slide film. A non-parametric χ^2 test was applied to analyse data from the dissection experiments.

Results

Bud formation

Buds, sometimes more than one at a time, emerge from the upper part of the polyp's column (Fig. 1). We propose to classify bud formation according to the sequence of eight developmental stages depicted in Figure 2:

Stage 1: Onset of budding recognizable as a slight protrusion of the endo- and ectoderm.
Stage 2: Protrusion more pronounced, angle between bud and body column >130°.
Stage 3: Bud assumes cuboidal shape, angle between bud and body column <130°.
Stage 4: Angle between bud and body column decreases to 90° or less; specialized future hypostomal cells become visible at the slightly pointed distal end of the bud.
Stage 5: The terminal area of future hypostome cells becomes larger; axial extension of bud.
Stage 6: Length of bud exceeds it's basal diameter; hypostomal cells start forming oral opening; usually five tentacle primordia

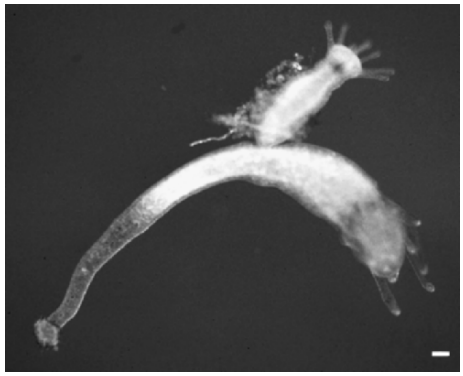

Figure 1. Carybdea marsupialis. Cubopolyp with bud ready to detach (stage 8). Scale bar, 100 μm (Photograph courtesy of Barbara Simmes).

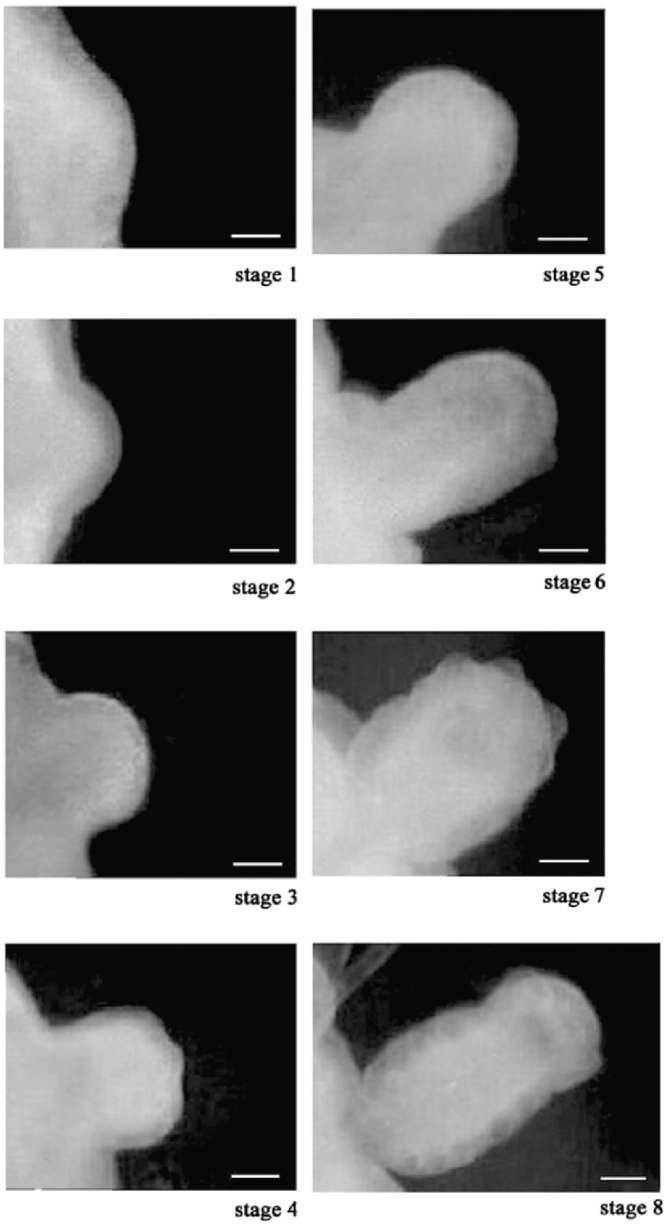

Figure 2. *Carybdea marsupialis*. Eight consecutive stages of bud formation, details described in the text. All scale bars, 100 μm.

appear; first autonomous contractions of the bud.

Stage 7: Bud diameter increased in the midgastric portion, a constriction appears at the basis; hypostome prominent with functional oral opening; tentacle primordia elongated.

Stage 8: Bud length increased, diameter decreased; bud-parent connection reduced to a narrow strand; a weak, necklike constriction separates gastric column from the ring of tentacle primordia encircling the bulbous hypostome. Bud ready to detach.

Under our laboratory conditions the entire bud-forming process may take 3–5 days. The fully differentiated buds separate actively by strong

contractions and by turns around the main body axis. Final bud length was found to vary between 500 and 1500 μm; larger parent polyps tend to produce larger buds.

The budding rate is positively correlated with the feeding frequency: Whereas a group of 30 polyps, fed once a week, produced a total of 25 buds within 12 days (i.e. an average of 2.1 buds per day), a second group of 30 polyps, fed 5× a week, developed a total of 156 buds (i.e. an average of 13 buds per day).

The budding rate is negatively correlated with illumination. The following observations were made on polyps already adapted to the different conditions: When exposed to the artificial light in the laboratory for 16 h, 60 polyps, fed once a week, produced 36 buds within 12 day (i.e. an average of 3 buds per day). The other group of 60 polyps, also fed once a week, was protected from light and propagated 63 buds (i.e. an average of 5.2 buds per day).

Further development of the buds

Once detached from the parent polyps, buds start creeping around, the oral end with the tentacles ahead ('creeping polyp stage,' Fig. 3a and b). Turns appear to be performed through stronger contractions and extensions. The vagile phase may last for some hours or up to several days, after which the individuals settle, contract, and firmly attach through adhesive material secreted by cells at the aboral pole (pedal disc). Then the young polyps become erect, resume growth, and increase the number of tentacles. We have followed up the development of one particular bud from separation through attachment until reaching the erect stage within a time period of 48 h (photomicrographs not shown). Active feeding usually starts with separation of the creeping polyp, but stage 7 and 8 buds were occasionally observed to catch and ingest live prey.

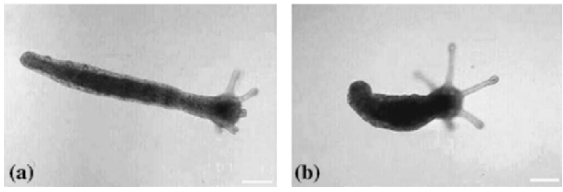

Figure 3. Carybdea marsupialis. The 'creeping polyp' stage of a bud recently detached from the parent, photographed in two phases of movement. Scale bars, 100 μm.

Though not analyzed statistically, observations of *C. marsupialis* in culture indicated that the time period until attachment of the creeping polyps tends to be considerably longer and to take several days in large, clean dishes provided with pasteurized seawater. However crowded conditions, very small containers, and dense biofilms on the bottom of the culture dishes promote settlement within hours. The asexual offspring settles often near parent polyps, thus creating small colonies (i.e. clones) of secondary polyps. Furthermore, creeping polyps seem to settle faster in the light than in the dark. This circumstantial evidence should prompt detailed investigations into presettlement behavior and into induction of irreversible attachment and polyp formation.

Regeneration

When adult, non-budding polyps were deprived of the oral part by sectioning below the tentacle crown, all fragments ($n = 30$) regenerated the ablated oral portion.

Different results were obtained following transection of adult, budding polyps during early phases of bud formation ($n = 50$ in each experiment):

Decapitation	Budding stage 1–2	Budding stage 2–3
Oral part regenerated, bud fully developed and separated	32%	78%
Oral part regenerated, bud developed but not separated (Y-shaped, double-headed polyp)	42%	22%
Oral part failed to regenerate, bud developed but not separated	26%	0%

The newly released creeping polyps exibited remarkable regeneration capacities: Within a group of 30 individuals bisected in the midgastric region, all 30 oral fragments regenerated the ablated portion either attached to the substratum or not. Of the 30 aboral fragments, two degenerated, but 28 regenerated an oral portion conforming with the original polarity. Within a group of 30 creeping polyps sectioned into three fragments of similar size (oral, midgastric, and aboral), all of the oral, tentacle-bearing fragments regenerated the ablated aboral portion. All of the 30 gastric fragments regenerated both the oral, tentacular region and the aboral part. However only 17 posterior isolates showed regeneration of the oral structures; the other 13 fragments degenerated.

Discussion

Previous studies performed on *Carybdea marsupialis* as well as on other cubozoans have focussed mainly on life-cycle features, on morphology, histology, ecology, taxonomy, and evolutionary relationships (e.g. Werner, 1984, for review). Special attention was paid to nematocytes because some of the medusae called 'box jellies' range among the most harmful cnidarians (Burnett, 1992; Golz, 1993; Avian et al., 1997; Simmes, 2002; Stangl et al., 2002).

Brief remarks on budding in species of the cubozoan genera *Tripedalia*, *Carybdea* and *Chironex* have been included in the papers by Werner et al. (1971), Cutress & Studebaker (1973), and Yamaguchi & Hartwick (1980). It appears that the budding mode is very similar: That of *Carybdea alata* (Arneson & Cutress, 1976) matches well with features described in this paper for *C. marsupialis*. The present account on bud morphogenesis and regeneration offers an approach to foster *C. marsupialis* as an experimental system for developmental studies. Easy to propagate in large numbers in the laboratory, most, potentially all, life-cycle stages are accessible. The proposed staging of the budding process, now added to the nine stages of medusa formation described in *C. marsupialis* by Stangl et al. (2002), may facilitate both future structural and developmental studies, and work applying molecular genetic techniques.

The solitary cubopolyps form buds in an apparently similar way and with the same polar organization as seen in species of the fresh-water hydrozoan *Hydra*, a traditional model organism for developmental studies (Galliot & Schmid, 2002, for review), thus offering the possibility for detailed comparative studies. Whereas *Hydra* spp. buds are released as fully differentiated young polyps readily attaching to the substratum, a stage named the creeping polyp is intercalated in *C. marsupialis* and other cubozoans. The type of locomotion is very similar to that observed in the non-ciliated frustules produced by budding of polyps in the taxon Limnohydrina. This creeping mode has been studied in *Gonionemus murbachi* in detail by Werner (1950). Subject of controversies for a long time, Werner (1950 for *G. murbachi*) and Reisinger (1957 for *Craspedacusta sowerbii*) demonstrated that frustules move with the future aboral pole of the polyp ahead, in contrast to the creeping polyps of the cubozoans which creep with the hypostome and tentacle anlagen in front. Creepers of *Tripedalia cystophora* thereby stretch out, like an antenna, one extremely long tentacle as the leading structure (Werner, 1984, Fig. 74B).

The impact of feeding on budding and on transformation into medusae respectively is remarkably different: whereas the budding rate increased with more frequent feeding (which was not unexpected), Stangl et al. (2002) observed that polyp metamorphosis into medusae is induced by food deprivation and can be prevented by daily feeding under otherwise permissive culture conditions.

Once detached from the parent polyps, it appears that some culture parameters, e.g. crowding, dense biofilms, speed up settlement of the creeping polyps, and final morphogenesis of the cubopolyp. Hartwick (1991) noted that creepers of *Chironex fleckeri* attached to various hard substrata with a bias towards vertical surfaces, indentations, and corners, suggesting a certain rugophila. However studies under strict axenic *in vitro* conditions to assess the possible role of surface texture, and of external biogenic cues for settlement, possibly emanating from biofilms on surfaces are not available yet, neither for *C. marsupialis* nor for any other cubozoan species (see Müller & Leitz, 2002 for a state of the art review on control of metamorphosis in cnidarians).

The ability of creeping polyp fragments to regenerate both the ablated oral and aboral portion matches well with the high bipolar regeneration capacities of frustules of *Vallentinia gabriellae* (Limnohydrina) reported by Auberson & Tardent (1980). It is, however, in obvious contrast to the strictly unidirectional restitution or regeneration of oral structures in fragments excised from larva-like propagules and polyps of the scyphozoan *Cassiopea* spp. (Curtis & Cowden, 1971; Neumann, 1977).

Many experimental studies have been addressed to 'head' regeneration (i.e. hypostome and ring of tentacles) induced concomitant with budding in *Hydra* spp. Müller (1996) points out that these processes are mutually exclusive and subject to a postulated secondary 'head' forming/inhibiting system (see also Galliot & Schmid, 2002, for review). Our observations on cubopolyps at early budding stages, which had been deprived of the 'head' region, point to the possibility that concurrent regeneration of the oral portion and bud formation (including separation) in *Carybdea marsupialis* are governed by a mutual control system as well. The pilot results presented here warrant extended comparative studies on both the experimental and theoretical level.

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Growth and age determination of the tropical Australian cubozoan *Chiropsalmus* sp.

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Key words: Cubozoa, Gompertz growth curve, statolith

Abstract

Chiropsalmus sp. medusae collected in this study ranged from 3 to 71 mm diagonal bell width and displayed growth best described by the following equation: size (mm) = 74.9 × exp (−exp(0.041 (time since metamorphosis (day) −35.6674))). Growth rates of up to 7 mm week^{-1} increase in diagonal bell width are theoretically possible, with animals able to reach sexual maturity in approximately 70 days. Correlation of the number of rings on the statoliths with the predicted age of the individual from the field produced a relationship that indicates the growth rings are laid down daily and as such could be used to infer age of the medusae. Over the 1998–1999 season, there were four influxes of juvenile cohorts, each occurring approximately 14 days after a major rainfall event.

Introduction

Cubozoans, or box jellyfish, are common inhabitants of Australia's tropical coasts during the warmer months of the year (Barnes, 1965, 1966; Brown, 1973; Hartwick, 1987, 1991; Burnett et al., 1996). Adult medusae die off at the end of the season (usually early May) with juvenile medusae budding from asexual polyps in November at the start of the season in at least *Chironex fleckeri* (Southcott) (Hartwick, 1991). Presently this is assumed to be similar for other Australian chirodropids, or multi-tentacled cubozoans. There is considerable confusion over the taxonomic identity of a second species, *Chiropsalmus quadrigatus* (Haeckel), also found commonly in these waters. These two species form the major component of the Australian chirodropids.

Descriptions of population structure in Australian chirodropids is limited to those of Barnes (1966), who indicated that, while cubomedusae are typically uniform in size within populations, marked differences in size are evident between populations. Juveniles originate asexually from polyps and increase in size rapidly (Hartwick, 1987, 1991; Hamner et al., 1995), reaching sexual maturity late in the season (Barnes, 1966; Hartwick, 1991); however, data quantifying these changes within populations are lacking. In comparison, data for scyphozoans are plentiful, and field studies have indicated that a typical growth pattern includes rapid growth to maturity, with growth at a decreased rate thereafter (Arai, 1997). This pattern is often best described by the von Bertylanffy growth curve (Schnute & Fournier, 1980; Gibbons & McCarthy, 1984; Pauly, 1984; Ryser, 1988; Bigelow et al., 1995; Mohammed, 1996; Platz et al., 1997); however, a logistic curve (Nigmatullin et al., 1995; Dimlich & Hoedt, 1998) or the Gompertz growth equation (Arkhipkin, 1997) may provide a better estimation of growth.

In parallel with a lack of data on population structure is a lack of a reliable means of aging

jellyfish. As many cnidarians are able to undergo growth and degrowth (Arai, 1997), the use of size to represent age in field populations may be inherently flawed. However, at least in one species of cubomedusae, daily growth rings found in the statoliths may prove to be a reliable indictor of age (Ueno et al., 1995, 1997), as it is in many species of fishes and cephalopods.

This paper examines the growth rate of *Chiropsalmus* sp., using modal progression of cohorts, to develop a growth curve and to determine if *Chiropsalmus* sp. has a single influx of juveniles per season or several. It also examines the validity of using growth rings of statoliths as a tool for determining the age of individual medusae.

Methods

The original description of *Chiropsalmus quadrigatus* describes the gonads as 'cox comb' in shape (Haeckel, 1879). The species of *Chiropsalmus* (Fig. 1) in Australia does not have 'cox comb' gonads, but has a cluster of eight grape-like gonads within the bell, suggesting it is probably a different species from that described by Haeckel; it is thus referred to as *Chiropsalmus* sp. in this study. Voucher specimens are housed at James Cook University, Cairns campus.

Specimen collection

Weekly sampling between early November 1998 and late April 1999 was done at Port Douglas, North Queensland, Australia, using a 30 m monofilament beach seine with a mesh of 10 mm. Sampling was conducted between the water's edge and 20 m from the shoreline, between 0600 and 0900 h. All *Chiropsalmus* sp. caught were removed and preserved in 4% seawater formalin. The diagonal bell width (dbw, the distance between diagonally opposite pedalia) of each specimen was recorded to the nearest millimetre with specimens in their natural, cuboidal shape. Five millimetre size classes were defined, with class 1 containing individuals 0–5 mm dbw.

Determination of cohorts

To determine if *Chiropsalmus* sp. has one or several influxes of juveniles into the population during the season, and whether influxes were correlated with rainfall, the number of individuals caught per 100 m of seine drag on each sampling occasion was calculated. Specimens less than 20 mm dbw were defined as juveniles. These data were plotted against time since 1 October, as was the amount of rain (recorded from Port Douglas meteorological station). The date at which rainfall

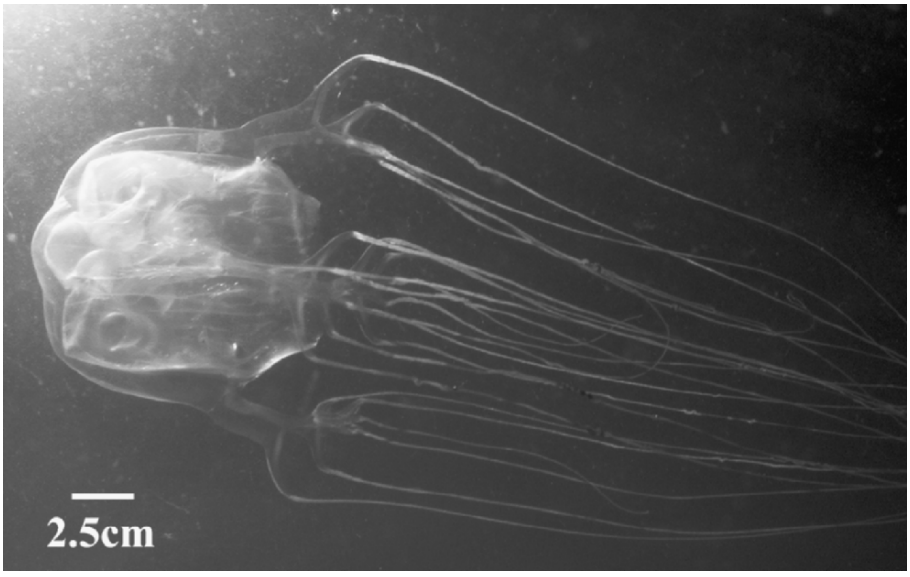

Figure 1. An adult specimen of *Chiropsalmus* sp., captured at Southern Four Mile Beach, Port Douglas, Australia.

was recorded was advanced 14 days to allow alignment of the peaks in cohort influxes with the peaks in rainfall.

Growth curve determination

Size-percent frequency histograms were produced. Modal class intervals were determined for each sample and, using the criteria described by Kaufmann (1981), the specific growth rate was calculated. Regression analysis of specific growth rate against both natural logarithm of the geometric mean dbw and reciprocal mean dbw was performed to determine the most appropriate growth curve. The resulting growth equation was calculated using a minimum medusae size of 1 mm, as Hartwick (1987) reported the size of newly produced medusae of a closely related species, *C. fleckeri*, to be between 1.2 and 1.4 mm immediately following metamorphosis. Regression analysis of the linear component of the resulting growth curve against time was used to produce a growth rate estimate.

Statolith ring correlation

Two statocysts from each of 24 individuals were removed. The statoliths were embedded in epoxy and ground using fine emery paper and toothpaste. Each statocyst was viewed under a light microscope using oil immersion and the number of rings in each statolith was counted (Fig. 2). The mean number of rings in the two statoliths (the difference was never $>5\%$) was regressed against the theoretical age of the medusa using the calculated growth equation. The use of tetracycline as a vital stain for statoliths caused rapid mortality in medusae.

Results

A progression through the modal size classes was observed, individuals becoming larger as the season progressed. Three distinct cohorts were observed over the 1997/1998 medusae season, with an additional influx having occurred prior to 9 November (Fig. 3). All four influxes occurred

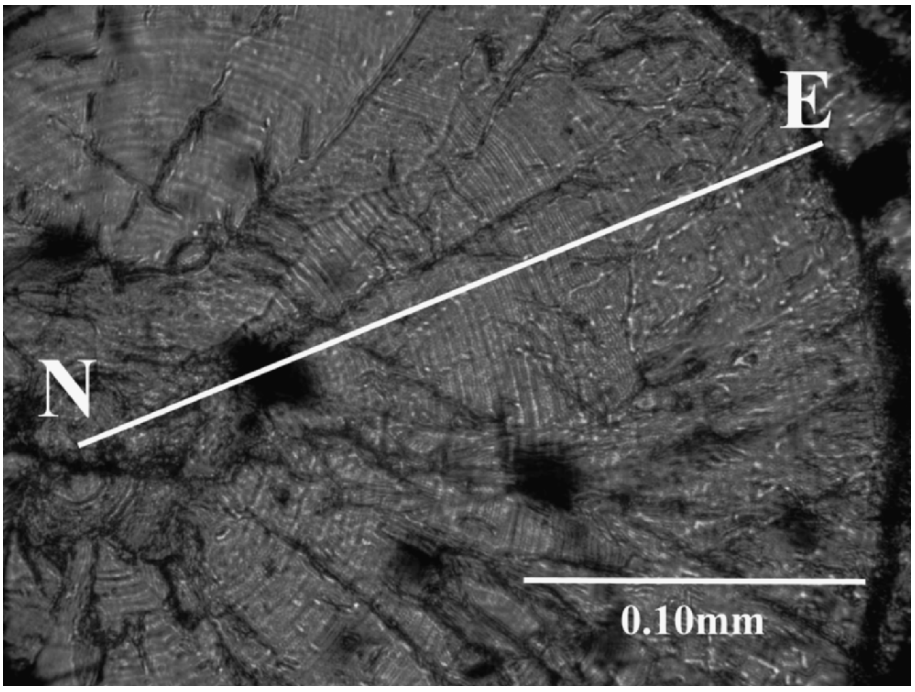

Figure 2. A sectioned statolith from *Chiropsalmus* sp. viewed through a compound light microscope. (N) nucleus or centre of statolith, (E) edge of statolith.

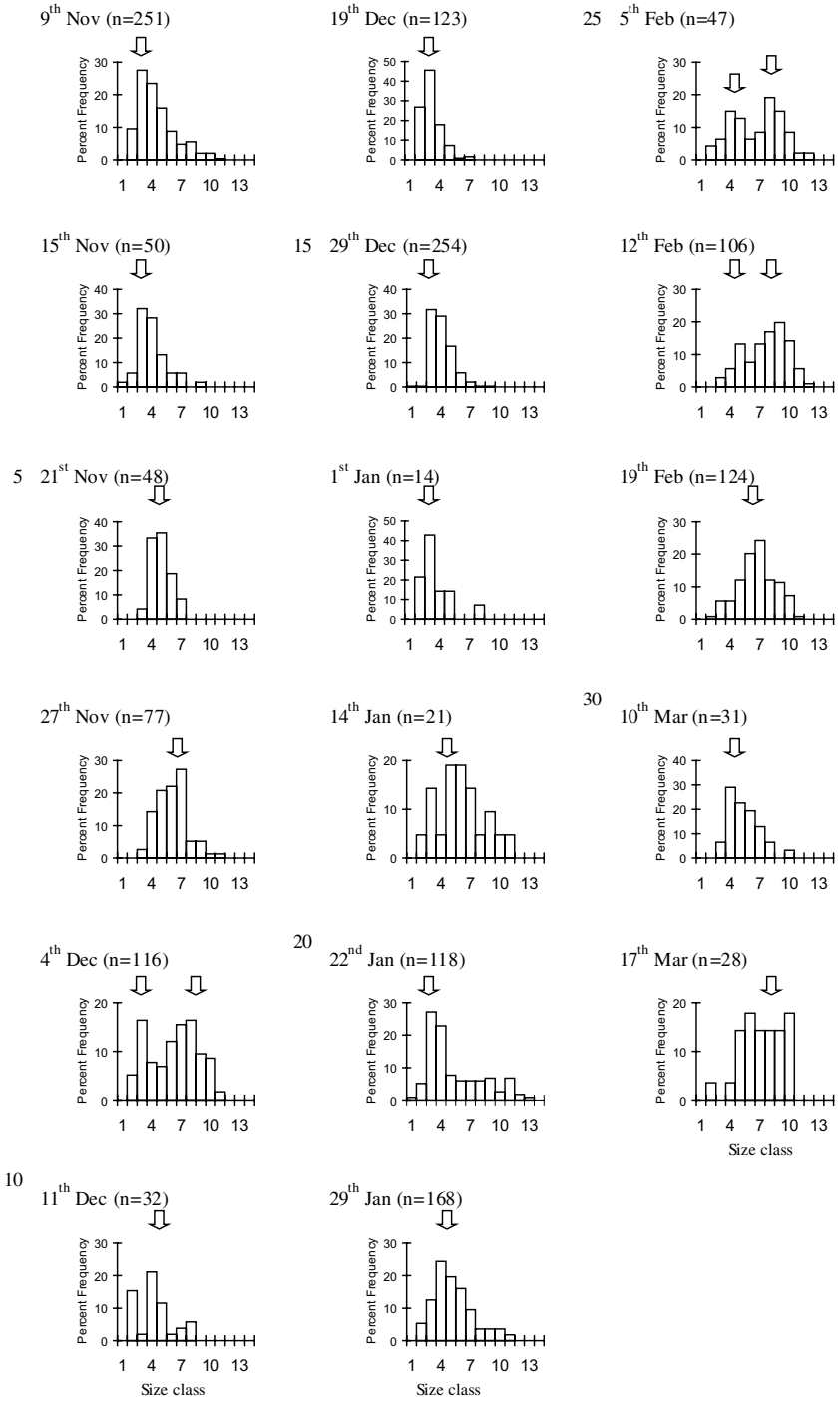

Figure 3. Modal progression of individuals of *Chiropsalmus* sp. sampled from Four Mile Beach, Port Douglas (⇩ = mode).

approximately 14 days after a rainfall event. Notably, no juveniles were found in October, even though a rainfall event occurred prior to that month. Likewise, from early April (day 182 onwards), no further influx of juveniles was recorded, even though a rainfall event occurred (Fig. 4).

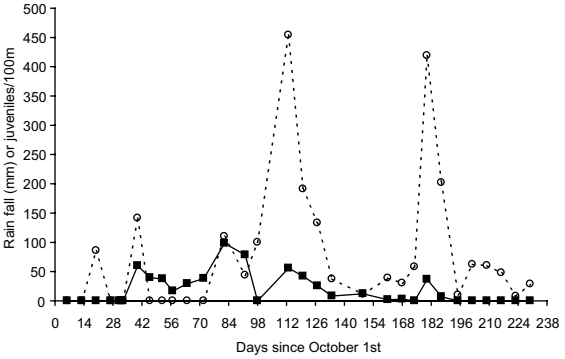

Figure 4. Recorded rainfall at Port Douglas, advanced 14 days, against time in days from October 1, 1998. Superimposed is the number (individuals/100 m) of juvenile (less than 20 mm diagonal bell width) *Chiropsalmus* sp. collected from Four Mile beach. (o) rainfall, (■) medusae numbers.

The specific growth rate of *Chiropsalmus* sp. was significantly correlated with both ln geometric mean size ($F = 33.29$, df $= 26$, $p < 0.001$) and reciprocal mean size ($F = 23.97$, df $= 26$, $p < 0.001$). However, ln geometric mean size explained more of the variation in specific growth rate ($r^2 = 0.554$) than ln geometric mean size ($r^2 = 0.469$). In accordance with the methods of Kaufmann (1981), a Gompertz rather than a von Bertylanffy growth curve was then fitted to these data. The Gompertz growth equation had the following form:

$$S = 74.9 \times e^{-e^{-0.041(t-35.6674)}},$$

where $S =$ size in mm at time t (in days) since metamorphosis (Fig. 5). The asymptotic (maximum theoretical) dbw for this species was calculated to be 74.9 mm. Regression analysis of the linear component of this relationship (from 30 to 70 days) ($F = 5960.95$, df $= 39$, $p < 0.001$) gave the maximum growth rate of *Chiropsalmus* sp. of 1 mm per day. Kinsey (1986) reported that the onset of sexual maturity occurs in this species at a mean size of 59 mm dbw, so, using the growth equation, medusae may reach sexual maturity after 72 days.

There was a significant and positive relationship between the theoretical age of the medusae and the number of growth rings ($F = 5.224$, df $= 1 \times 22$, $p = 0.032$, Fig. 6). The equation of this relationship was

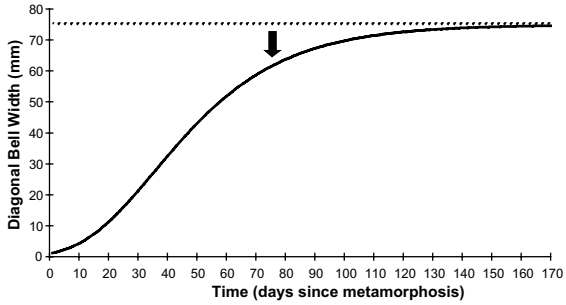

Figure 5. Gompertz growth curve for *Chiropsalmus* sp. showing the onset of sexual maturity and asymptotic diagonal bell width. ⬇ mean size (mm diagonal bell width) at sexual maturity (Barnes in Kinsey, 1986), (.....) theoretical asymptotic diagonal bell width.

$$\text{number of rings} = 0.688 \times \text{dbw(mm)} + 9.89.$$

The slope of this relationship was not significantly different from 1 (95% confidence limits = 0.064–1.313).

Discussion

The growth of *Chiropsalmus* sp. medusae was rapid, with a maximum of 7 mm in 1 week during the summer of 1998/1999 (when mean sea water temperature was 28.6 °C). While this maximum growth rate estimate is comparable to that Guest

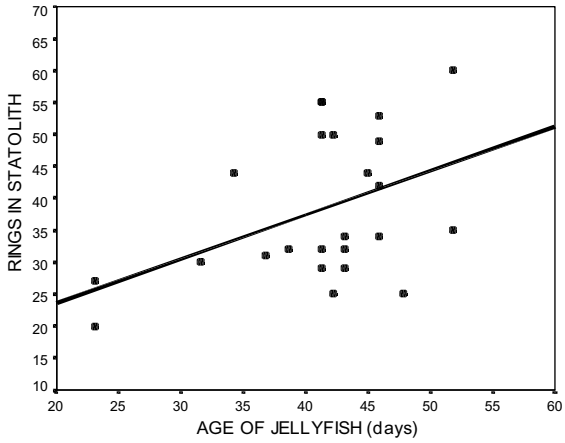

Figure 6. Relationship between the number of rings in the statolith of *Chiropsalmus* sp. and the theoretical age of the organism based on diagonal bell width. Number of rings = 0.688 × diagonal bell width (mm) + 9.89 ($F = 5.224$, df $= 1 \times 22$, $p = 0.032$).

(1959) provided for *Chiropsalmus quadrumanus*, it is 7–14× higher than that established for scyphozoans in similar field studies (Olesen et al., 1994; Arai, 1997).

At this rate of growth, individuals of *Chiropsalmus* sp. can reach their theoretical maximum size of approximately 75 mm dbw within 130 days, well within the average length of the medusa season of approximately 180 days (Gordon, 1998). This maximum theoretical size compares favourably to maximum size of field-collected animals reported by Barnes (1966), Sutherland (1983), and Halstead et al. (1990). Maximum sizes of 200 and 100 mm reported by Sutherland (1994) and Rifkin (1996), respectively, appear theoretically inaccurate based on these data. In these cases, specimens may have been confused with *C. fleckeri*, which is morphologically very similar but grows significantly larger (Southcott, 1960; Barnes, 1965, 1967; Keen, 1971; Marsh et al., 1986; Fenner & Williamson, 1996).

The relationship between number of rings in the statolith and theoretical age of the medusae not being significantly different from one suggests that any increase in age corresponds with a similar increase in growth rings. That is, the growth rings appear to be laid down daily. This was shown by Ueno et al. (1995, 1997) in another cubomedusa, *Carybdea rastoni*. The ability to predict the age of the medusae from the number of growth rings is important for comparisons between populations with differing environmental temperature and food resources, as size of medusae is not a reliable indication of age.

In contrast to *C. fleckeri*, with a life span thought to be the length of the medusa season (Hartwick, 1991), a number of generations of *Chiropsalmus* sp. may occur within one season. This is supported by the data collected in this study, with four influxes of juveniles during a season. The time to sexual maturity for *Chiropsalmus* sp. (72 days) is longer than the interval between juvenile influxes (~40 days). Therefore, juveniles in successive influxes cannot have arisen from the reproduction of sexually mature individuals of the previous influx. Alternatively, polyps derived from the different cohorts may produce juveniles at different times of the season. Unlike scyphozoans, which undergo strobilation and produce several medusae per polyp, cubozoans are characterised by the production of only one medusa from a polyp. Consequently, the production of separate cohorts cannot be produced by continual liberation of medusae. It appears that different polyps produce medusae at different times, resulting in distinct cohorts throughout the season.

The polyps may be trading off between the potential benefits and associated hazards of undergoing metamorphosis at a particular stage of the season. What these advantages and disadvantages are is undetermined, but appear to be associated with salinity and possibly water temperature changes, as juvenile influxes show a strong relationship with rainfall events, which often lead to large variations in salinity and temperature of estuaries, where the polyps are thought to be located. To test this hypothesis, studies need to be conducted on the polyp stage of the life cycle, but unfortunately the geographic location of this species' polyps is still unknown.

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The life cycle of *Chrysaora lactea* Eschscholtz, 1829 (Cnidaria, Scyphozoa) with notes on the scyphistoma stage of three other species

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Key words: Semaeostomeae, Pelagiidae, Brazil

Abstract

The life cycle of *Chrysaora lactea* Eschscholtz, 1829, a common species on the Brazilian coast, is described. Mature medusae were collected and isolated in a *planktonkreisel*, whereupon planulae appeared after 1–2 days. These planulae settled and metamorphosed into polyps. Fully developed scyphistomae typically possessed 16 tentacles, and on strobilation produced from 2 to 10 ephyrae. The ephyrae were transparent and had characteristic nematocyst warts on the exumbrella. Tentacles first appeared near the margin on the subumbrella. Ephyrae and young medusae were maintained in laboratory conditions up to 7 months.

Introduction

The scyphozoan medusa *Chrysaora lactea* Eschscholtz, 1829 is considered widespread along the Brazilian coast (Migotto et al., 2002). Despite its wide range, from Cuba to Argentina (Mayer, 1910; Mianzan & Cornelius, 1999), few records of the species appear in the literature since its original description by Eschscholtz (1829), based on Brazilian material from Guanabara Bay (Rio de Janeiro).

The life cycle of the species is unknown, and little information on the biology of the species exists. Agassiz & Mayer (1898) commented on the habits of the species in Havana Harbor, Cuba. Mianzan (1989a, b) mentioned the capture of some ephyrae in plankton samples from the Argentinian coast. Tronolone et al. (2002) reported the occurrence of an ephyra on the Brazilian coast (São Sebastião Channel) and described its early development.

All members of the family Pelagiidae Gegenbaur, 1856 whose life cycles are known possess scyphistomae (see list below and Table 1 in Tronolone et al., 2002) except *Pelagia noctiluca* (Forskål, 1775), which has a holopelagic life cycle without a polyp stage (Goette, 1893; Russell, 1970; Rottini Sandrini & Avian, 1983). Pelagiidae with known life cycles are *Chrysaora achylos* Martin, Gershwin, Burnett, Cargo & Bloom, 1997 (Schaadt et al., 2001); *Chrysaora colorata* (Russell, 1964) (Gershwin & Collins, 2002); *Chrysaora fuscescens* Brandt, 1835 (not formally described but mentioned by Gershwin & Collins, 2002); *Chrysaora hysoscella* (Linnaeus, 1766) (Delap, 1901); *Chrysaora melanaster* Brandt, 1835 (Kakinuma, 1967); *Chrysaora quinquecirrha* (Desor, 1848) (Littleford, 1939; Calder, 1972); and *Sanderia malayensis* Goette, 1886 (Uchida & Sugiura, 1978).

Where known, the cnidom of species of *Chrysaora* conforms to that of other Scyphozoa, the nematocyst types being holotrichous isorhizas and heterotrichous microbasic euryteles (Russell, 1970; Calder, 1971, 1983; Arai, 1997).

Table 1. A comparison of the early stages of three Atlantic species of *Chrysaora* (*C. hysoscella*, *C. lactea*, and *C. quinquecirrha*) and one from the north Pacific (*C. melanaster*)

Stage	*Chrysaora* species	*hysoscella*	*lactea*	*quinquecirrha*	*melanaster*
	Tentacle number	14–16	12–21	13–20	16–18
	Color	Whitish-orange	Whitish	Pinkish	Whitish
Scyphistomae	Max. height (mm)	2.5	1.2	1–4 (3.75)	2–3.2
	Stalk height (mm)	0.25	0.46	1.5	0.2
	Stalk/max. height	0.1	0.38	0.37	0.06
	Width oral disc (max.)	1.75	0.86	0.6–1.5	1.8
Podocysts	Diameter (mm)	0.25–0.44	0.2–0.3	0.35–0.45	0.3–0.4
	Number/strobila	4–6	2–10	15	20–30
Ephyrae	Color	Pale orange	Transparent	Pinkish	Pinkish
	Liberated size (mm)	1.62–2	1–2	2.45–2.75	2–3

The data presented in the table are a combination of observations of specimens in cultures and from literature records (Littleford, 1939; Kakinuma, 1967; Russell, 1970).

The aim of this study was to describe the life cycle of *Chrysaora lactea* based on observations of laboratory cultures.

Material and methods

Life cycle studies began with the collection, by hand net, of mature medusae from surface waters in the Cananéia estuarine system (25° S, 48° W), São Paulo state, southeastern Brazil. Polyps were obtained by isolating mature medusae on two occasions (November 2000 and January 2003). On both occasions the collected medusae (males and females) were placed together in a *planktonkreisel* (built according to Greve, 1968) for a week. Release of gametes was observed and planulae appeared after 1–2 days. These planulae settled in the middle tube of the *planktonkreisel* and on watch glasses placed on the bottom. After some time, these were transferred to culture dishes. The subsequent cultivation was similar to methods described for coronates by Jarms et al. (2002), with minor adaptations for the species. Polyps, ephyrae, and young medusae were fed with *Artemia franciscana* Kellogg, 1906 nauplii. Ephyrae and young medusae were also fed with ephyrae of the coronates *Nausithoe aurea* Silveira & Morandini, 1997 and *Nausithoe maculata* Jarms, 1990 as well as gonad pieces of the clam *Perna perna* Linnaeus, 1767.

We used specimens of other *Chrysaora* species in culture for the comparison of early stages (polyps and ephyrae). These included *C. hysoscella* (Linnaeus, 1766) polyps, from planulae released by medusae collected by the first and last authors in Helgoland, Germany (North Sea); *C. melanaster* Brandt, 1838 (Zoo-Aquarium Berlin); and *C. quinquecirrha* (Desor, 1848) (Zoo-Aquarium Berlin). The later two species were from the last author's cultures. Cultures were kept in controlled temperature chambers at 22 °C, except those of *C. hysoscella*, which were kept at 15 °C.

Nematocyst types were determined from live specimens using a Wild M20 light microscope. Measurements were undertaken based on 30 undischarged capsules. Nematocyst nomenclature followed that of Mariscal (1974).

Different life cycle stages were observed and photographed using a Zeiss Stemi SV11 stereomicroscope and a Zeiss Jenaval mf-AKS microscope, or in a small aquarium with a black background using a Minolta reflex camera.

Specimens of polyps, ephyrae, and young medusae were deposited in the cnidarian collection of the Museu de Zoologia, Universidade de São Paulo (MZUSP), the numbers being mentioned in the text.

Results

Life cycle

Collected medusae released gametes, and fertilized eggs developed into planulae. These planulae settled and metamorphosed into early polyps. These polyps grew and, reproduced asexually, producing podocysts, and strobilated, liberating ephyrae. Ephyrae grew to mature medusae. Our observations thus demonstrate that the species undergoes a metagenetic life cycle.

Description of stages

Planula

The planulae are pear-shaped and elongated along the anterior–posterior axis. Their lengths range from 0.14 to 0.2 mm, and widths from 0.06 to 0.09 mm. They are whitish in color. After 1–2 days swimming, the planulae move close to the bottom. There they attach themselves to the substrate by their anterior ends. Usually they attach on the underside of plastic and glass substrates.

Scyphistoma

After one-two days of settlement, the primordia of two tentacles can be seen, as well as the mouth opening. After 12–24 h the next two tentacles appear. The mouth begins to elevate from the oral disk. At this stage the young scyphistoma has a thin stalk surrounded by a periderm (Fig. 1). The size of the observed scyphistomae was 0.1–0.18 mm in oral disk diameter and 0.14–0.2 mm in height. The four-tentacled stage can last for up to 75 days. Normally after 6–10 days the rudiments of the next four tentacles appear, almost simultaneously, between the first ones. At the eight-tentacled stage the polyp starts to produce some podocysts. The thin stalk still continues at this stage, but with development the stalk becomes thicker and almost indistinct from the calyx. At this stage the observed polyps were 0.2–0.36 mm in oral disk diameter and 0.4–0.6 mm in height. The typical number of tentacles is 16, but it can be variable (12–21). The tentacles are extremely extensible, up to five times the polyp height. The mouth is cruciform, with prominent lips somewhat elevated above the oral disk. The four gastric septae are indistinct in young scyphistomae, but in larger ones they became more visible. The polyps are whitish to cream in coloration (Fig. 2), up to 1.2 mm. Some scyphistomae were deposited under the number MZUSP 0365.

A comparison of the early life cycle stages (polyps and ephyrae) of three Atlantic species of *Chrysaora* and a Pacific one are presented in Table 1.

Podocysts

The podocysts were produced continuously by the polyps (Fig. 3), but their numbers appeared to have been greater when no food was administered to the polyp culture dish. They are trapezoid in form (side view) with the apex slightly convex. The diameter ranged from 0.2 to 0.3 mm and the height from 0.08 to 0.1 mm. The color is yellowish-brown. None of the podocysts regenerated polyps.

Strobila

Polyps initiate polydisc strobilation by forming a single disc just below the oral disc. With time,

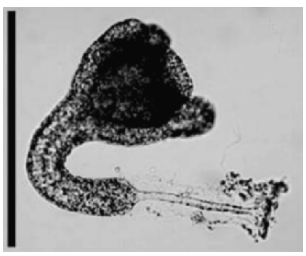

Figure 1. Newly settled polyp of *Chrysaora lactea* Eschscholtz, 1829. Note the primordia of the first four tentacles, elevation of the mouth cone, and the thin periderm surrounding the polyp stalk. Scale, 0.1 mm.

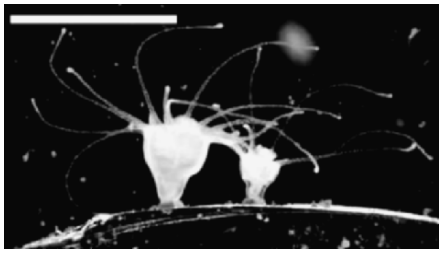

Figure 2. Five-month-old polyps of *Chrysaora lactea* Eschscholtz, 1829. Scale = 2 mm.

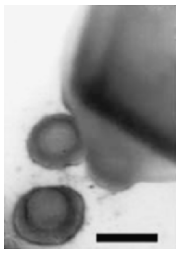

Figure 3. Detail of the basal portion of a polyp of *Chrysaora lactea* Eschscholtz, 1829, showing two podocysts. Scale, 0.2 mm.

several discs appear below the first one, towards the aboral part of the body (Fig. 4). The 'head' gradually resorbs the tentacles and differentiates into the uppermost ephyra. The lower discs differentiate slower than the first ones (Fig. 5). The tentacles of the polyp transform into the rhopalia of the distal ephyra. The first ephyra is liberated before the complete formation of the last one. The ephyrae chain shows contraction movements in the strobila. The strobilation chain is whitish as is the remaining basal polyp. Before the release of the last ephyra, the polyp regenerates new tentacles. The whole process, at 22 °C temperature, takes about 10 days (from the beginning of the first disc to the release of the last ephyra). A given strobila produced from 2 to 10 ephyrae at a time.

Ephyra

The ephyra (Fig. 6) typically had eight arms (lobes); 16 pointed marginal lappets; eight rhopalia, with light yellowish concretions; a cruciform mouth; diameter 1–2 mm just after liberation; a transparent body; and the characteristic nematocyst clusters (Russell, 1970) of the *Chrysaora* pattern (Gershwin & Collins, 2002). Some ephyrae were deposited under the number MZUSP 0363-0364.

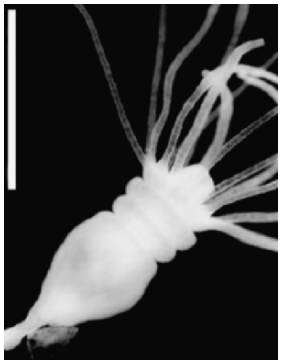

Figure 4. Early strobila of *Chrysaora lactea* Eschscholtz, 1829. Scale, 1 mm.

Figure 5. Advanced strobila of *Chrysaora lactea* Eschscholtz, 1829, showing development of chain of ephyrae at different stages. Scale, 1 mm.

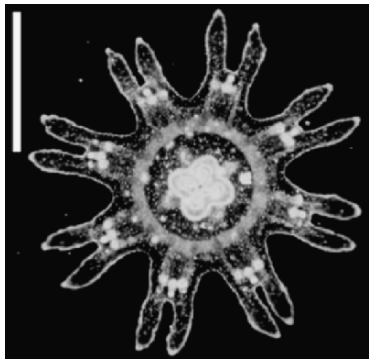

Figure 6. Oral view of newly liberated ephyra of *Chrysaora lactea* Eschscholtz, 1829. Note the pair of nematocyst clusters at the base of each marginal lobe (adapted from Tronolone et al., 2002). Scale, 0.5 mm.

Young medusa

Ephyrae from polyp cultures – The most striking feature is that all tentacles originate from the subumbrella close to the margin of the bell. The first tentacles (primary) begin to develop from the clefts between the rhopalar lappets, and they start growing almost simultaneously 3–6 week after strobilation (Fig. 7). The secondary tentacles develop from the subumbrellar portion of the rhopalar lappets, which divides, later (3–6 months) forming two new lappets each. These tentacles do not develop at the same time. With development, the tentacles occupy a position at the margin of the

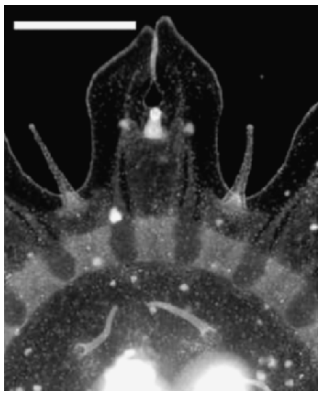

Figure 7. Oral view of ephyra of *Chrysaora lactea* Eschscholtz, 1829, showing development of the primary tentacles. Note that tentacles develop from the subumbrella near the margin of the ephyra. Scale, 1 mm.

animals. Young medusae were kept in cultures for up to 7 months (Fig. 8). Two young medusae were deposited under the numbers MZUSP 0361-0362.

Medusa

Adult (mature) medusae (Fig. 9) conform with the re-description of the species from Jamaica in Mayer (1910: 583–584, Fig. 369) the variation in tentacle number per octant, eventually in the same animal, being noteworthy. Development of the tentacles per octant, from one to five, is in agreement with Mianzan & Cornelius (1999).

Nematocysts

The cnidom of the medusa and polyp stages is composed of holotrichous isorhizas and heterotrichous microbasic euryteles; the ephyrae stage possessed only holotrichous isorhizas. Measurements are presented in Table 2. Holotrichous isorhizas are present in all life cycle stages, but the large rounded holotrichous isorhizas are present only on the tentacles of the medusa and in the nematocyst clusters of the ephyra. Contact with the medusa tentacles produces a mild sting that lasts 30–60 min.

Figure 8. Juvenile medusa of *Chrysaora lactea* Eschscholtz, 1829, 7 months after being liberated from the strobila. Scale, 3 cm.

Figure 9. Adult medusa of *Chrysaora lactea* Eschscholtz, 1829, just after collection and with three tentacles per octant. Scale, 5 cm.

Discussion

In the past, species of *Chrysaora* having 40 tentacles (up to five tentacles per octant) were assigned to *Dactylometra* (Agassiz, 1862). However, subsequent research on development of certain *Dactylometra* species led systematists to consider it congeneric with *Chrysaora*, because *Dactylometra* species may attain sexual maturity before the total number of tentacles has developed (i.e., at the *Chrysaora*-stage). Recently, phylogenetic analysis of the family Pelagiidae, as proposed by Gershwin & Collins (2002), indicated that species formerly assigned to *Dactylometra* formed a separate group. They commented that analysis of additional characters may support *Dactylometra* being a clade in the future. Until now, the only differences between the two nominal genera are the relative number of lappets and tentacles in adult medusae.

Almost all scyphozoans in which the life cycle is known possess a polyp stage (Tronolone et al., 2002). As in other species of the genus *Chrysaora*

Table 2. Measurements in micrometers (μm) of nematocyst types of different stages of *Chrysaora lactea* Eschscholtz, 1829. holotr. isorhiza, holotrichous isorhiza; het. microb. euryt., heterotrichous microbasic eurytele

Stage	Location	Nematocyst type	Length	Mean ± SD	Width	Mean ± SD
Medusa		holotr. isorhiza	14.4–18	16.18 ± 1.05	9.6–12.6	11.66 ± 0.73
	Tentacles	het. microb. euryt.	10.8–13.8	11.8 ± 0.72	6.6–10.2	7.9 ± 0.91
		het. microb. euryt.	6–8.4	7.12 ± 0.64	3.6–4.8	4.1 ± 0.41
	Gastric filaments	holotr. isorhiza	4.2–6	5.26 ± 0.58	2.4–4.2	3.44 ± 0.62
		het. microb. euryt.	12–15.6	13.92 ± 1.09	8.4–12	10.74 ± 1
Ephyra	Clusters	holotr. isorhiza	10.8–13.2	11.56 ± 0.58	9–11.4	10.06 ± 0.88
	Umbrella	holotr. isorhiza	4.8–6.6	5.74 ± 0.49	2.4–4.2	3.5 ± 0.61
Scyphistoma		holotr. isorhiza	5.4–7.2	6.2 ± 0.45	3–4.8	4.1 ± 0.52
		het. microb. euryt.	9–12	10.62 ± 0.97	6.6–9	7.86 ± 0.79

(Delap, 1901; Littleford, 1939; Kakinuma, 1967; Calder, 1972; Uchida & Sugiura, 1978; Schaadt et al., 2001; Gershwin & Collins, 2002), *C. lactea* has a polyp stage and a metagenetic life cycle.

Newly settled scyphistomae of *C. lactea* have a thin stalk, observed also in *C. melanaster* (Kakinuma, 1967) but otherwise not mentioned in the literature. Budding was not observed in our cultures of *C. lactea*. Where polyps of *C. lactea* occur in nature remains unknown. In the area where medusae were collected, several possible substrates (rocks, sea grasses, oyster shells) were inspected but polyps were not found. A 1-year experiment with fouling ceramic plates, sampled monthly, was undertaken near the area where the medusae were collected but no scyphistomae were found. A comparison between known polyps of *Chrysaora* showed that *C. quinquecirrha* and *C. lactea* have a longer stalk than in the other species.

Ephyrae were found only occasionally (Mianzan, 1989a, b; Tronolone et al., 2002), but medusae are rather common and supposedly widespread along the Brazilian coast (Migotto et al., 2002). Ephyrae have the characteristic nematocyst clusters that distinguish *Chrysaora* from other Pelagiidae (according to Russell, 1970; Gershwin & Collins, 2002). Ephyrae of *Sanderia malayensis* have nematocyst clusters in a more disorganized distribution, although there is also a pair of clusters at the base of each marginal lobe (Uchida & Sugiura, 1975). Early development of the ephyrae agrees with the description by Tronolone et al. (2002) for the species. Differences were noted in the time of development of the tentacles, but we believe that these are due to differing cultivation conditions. An interesting character is that the primary tentacles of the medusa begin to develop on the subumbrella near the margin, and that with growth of the animals they rest in the clefts between the lappets. Secondary tentacles begin development under the lappets on the subumbrella (see Fig. 7 in Tronolone et al., 2002) and with growth the lappets split, 'creating' spaces for the tentacles, as described for *C. hysoscella* (Russell, 1970).

As pointed out by several researchers in other *Chrysaora* species (Delap, 1901; Calder, 1972; Sommer, 1988, 1992, 1993; Lange & Kaiser, 1995; Olesen et al., 1996; Schaadt et al., 2001; Raskoff et al., 2003), the ephyrae and medusae of *C. lactea* voraciously eat gelatinous zooplankters, and specially other medusae.

Some authors have described nematocysts in other species of *Chrysaora,* e.g. *C. hysoscella* (Russell, 1970), *C. quinquecirrha* (Calder, 1971, 1977), and *C. achlyos* (Radwan et al., 2000). The nematocyst cluster of ephyrae, composed of holotrichous isorhizas in *C. lactea*, was also noted by Calder (1972) in *C. quinquecirrha*, and Russell (1970) commented that this type was more abundant in the lobes of *C. hysoscella*.

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D-Methionine and gold chloride alleviate adverse effects of glutamate on motility of ephyrae of *Aurelia aurita* (Linnaeus, 1758) (Scyphozoa: Semaeostomeae)

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Key words: jellyfish, pulsing, swimming

Abstract

Glutamate (MSG) causes low pulse numbers and swimming cessation in *Aurelia* jellyfish ephyrae. Ephyrae given MSG for 1 h and subsequently maintained in artificial sea water (ASW) were observed at 1, 3, 24, and 48 h intervals. Abnormality of motility was found at all post-treatment periods but some ephyrae resumed swimming and normal pulsing within 48 h. Swimming and pulsing were impaired in a significant number of ephyrae within 15 min of MSG treatment. The mechanism of MSG action on ephyrae motility is unknown, but glutamate damage to neurons and hair cells of higher animals is partly attributed to the formation of reactive oxygen species (ROS). Laser confocal fluorescent microscopy of ephyrae following MSG treatment indicated an increase of calcium and free radicals in the ephyrae as early as 5 min following MSG exposure. To determine whether antioxidants could alleviate MSG effects, we exposed ephyrae to gold chloride before, during, and after treatment with MSG. Ephyrae given gold chloride pre-treatment for 1 h and then transferred into gold chloride plus MSG for 1 h showed statistically significant recovery from MSG impairment of pulsing at the 3, 24, and 48 h post-glutamate time periods and higher numbers of swimmers at 3 h and 24 h. Ephyrae groups given gold plus MSG but without gold pretreatment showed recovery of swimming at 24 h and pulsing at 48 h. D-methionine given simultaneously with MSG significantly improved the pulse numbers and swimming of ephyrae at the 3, 24, and 48 h post-glutamate time periods compared to those receiving MSG alone. Both D-methionine and gold chloride accelerated the time of recovery from glutamate-induced motility impairment, possibly through their antioxidant activities.

Introduction

In mammals, including humans, a cascade of events generated by glutamate excitotoxicity is involved in brain and spinal cord trauma, aging, and neurodegenerative diseases, including stroke, ALS, Parkinson's, and Alzheimer's diseases. One of the earliest events in glutamate excitotoxicity is the modification of glutamate receptors, including *N*-methyl-D-aspartate (NMDA), which results in an influx of calcium into neurons followed by other cellular events leading to neuronal necrosis and/or apoptosis. The generation of free radicals including reactive oxygen species (ROS) following excessive glutamate exposure is believed to play an important role in the subsequent neuronal damage.

Very little is known concerning the effects of excessive glutamate on lower organisms, especially the coelenterates. We therefore use tiny ephyrae of *Aurelia aurita* (Linnaeus, 1758) to study effects of extraneously introduced monosodium glutamate (MSG) on their pulsing and swimming motility. Ephyrae respond rapidly, adversely, and consistently to glutamate treatment, allowing study

of compounds that may prevent such responses. To determine whether substances which reportedly have free radical scavenging abilities will negate the adverse effects of glutamate, we introduced gold chloride and (separately) D-methionine with glutamate to the ephyrae. Further, using fluorometric dyes and laser confocal microscopy, we endeavored to demonstrate whether there is an increase in intracellular calcium activity and the generation of increased free radicals in the ephyrae shortly after their exposure to glutamate.

Material and methods

Testing

At least three experiments were done at different times for each of the compounds tested, using two-four ephyrae per test group per experiment. Polyps were exposed to 10^{-5} M iodine in artificial sea water (ASW) to produce ephyrae through strobilation of the polyps (Spangenberg, 1967). Test ephyrae were selected at least 2 days after release from their strobilae when they are able to swim well. All test compounds were dissolved in the ASW used to culture the jellyfish (Spangenberg, 1965). Ephyrae were placed in test solutions in new glass test tubes, previously washed in distilled water, and dried. Each tube held one ephyra. The ephyrae were maintained at room temperature (16–19 °C) and were not fed during the testing.

Compounds tested

Tests were done to determine the tolerance of ephyrae to MSG using a series of MSG concentrations in ASW. From these tests, the concentration of 5 mM MSG was selected because it elicited the most consistent response from the jellyfish, was non-lethal, and permitted recovery with time. Similarly, a series of tests was done to determine the appropriate dosage of gold chloride (0.022 μM) and D-methionine (5 mM) for use in conjunction with MSG. All chemicals were purchased from Sigma (St. Louis, MO).

At intervals of baseline (prior to addition of test compounds), 1 h in the test compound, and 1, 3, 24, and 48 h after removal from the test compound, the test was read as follows: Each test tube was inverted to bring the ephyra into the upper portion of the tube and the ephyra was observed for one minute. The number of pulses per minute was counted using a manual counter and the animal's swimming behavior was noted and recorded. Ability to swim was defined as the ability of the ephyra to move with direction along linear or curvilinear, but not circular pathways, against current or gravity. A normal pulse was defined as a rapid simultaneous contraction of all lappets downward at an angle of >80°, resulting in movement of the ephyra.

Statistics

Test results were subjected to the Student's paired *t*-test and the nonparametric Rank Sum test to determine statistical significance, with $p < 0.05$. Sample number (n) ranged from 6 to 60 ephyrae.

Laser confocal fluorescent microscopy studies

Laser confocal fluorescent microscopy was used to detect calcium and free radicals in the ephyrae during the first hour after exposure of the ephyrae to MSG. We evaluated several visible light calcium indicators, including fura red, fluo-3, and rhod-2. Fluo-3 and rhod-2 were suitable, but fura red had poor cellular loading characteristics and was not used. The free radical indicator 5-(and 6-)carboxy-2′,7′ dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA), which is sensitive to peroxide, hydroxyl, and nitric oxide (NO) among other ROS, was also used, as was DAF-FM diacetate (4-amino-5-methylamino-2′,7′-difluorescein), a more specific NO indicator. All fluorescent reagents were obtained from Molecular Probes (Eugene, OR). Combinations of 5 μM fluo-3, 5 μM rhod-2 AM (acetoxymethyl ester), 10–20 μM carboxy-H2DCFDA, and 10 μM DAF-FM diacetate with 0.01% Pluronic 127 in ASW were incubated with ephyrae for 1–2 h at 22 °C in glass vials in the dark, with approximately 1 ml solution per ephyra. After transfer into ASW and washout of the residual indicator for 15–30 min, ephyrae were placed on a petri dish with a central well with a 12 mm glass cover slip bottom (World Precision Instruments, Sarasota, FL) and were restrained with a fine nylon mesh held over the ephyrae using a stainless steel washer. This

arrangement restricted the movement of the animals and also permitted exchange of bathing solutions. The ephyrae were not impaired by the treatments as determined by both microscopic measurements and motility tests. Temperature during these experiments was 22–25 °C.

Fluorescence measurements were made using a Zeiss 510 laser confocal microscope. Appropriate excitation wavelength and emission filter and dichroic combinations permitted simultaneous measurements of intracellular calcium, ROS, and NO using fluo-3, carboxy-H2DCFDA, and DAF-FM diacetate, respectively (green images collected at 488 nm excitation, 505–550 nm emission) and intracellular calcium activity (red images collected at 543 nm excitation 560–615 nm emission) using rhod-2, as well as single wavelength experiments with individual indicators. Pinhole settings were nominally 1 Airey unit for the selected objective. Microscope objectives used are listed in the figure legends. An ASW solution containing 10 μM ionomycin was used to saturate the calcium indicators at the end of the experiment.

Using a Zeiss 10×, 25×, or 40× Plan NeoFluor lens, 2–5 μm optical slices of the lappet and rhopalia were taken. Depending on the thickness of the ephyrae or rhopalia and the optical section, 15–30 512 × 512 pixel, 1024 bit resolution images were collected per time point and stored as multi-image TIFF files. Fluorescence for each indicator was measured using the Metamorph image processing program (Universal Imaging, Downingtown, PA) by first color separating the images and then determining the intensity of six areas whose position was maintained constant during the experiment. This system permitted confocal measurements of ephyrae using low power (to 40× objectives) but was not suitable for higher magnifications due to the large working distance caused by the well slide's and ephyrae's thickness.

Results

Glutamate dosage range

Testing a range of MSG from 0.5 to 5 mM revealed that ephyrae tolerated a dosage of 5 mM for up to 24 h. As compared with baseline and same time period controls, ephyrae given 5 mM for 24 h were more severely impaired with regard to both pulsing and swimming ability than those receiving lower dosages (data not shown). Further testing revealed that exposure to 5 mM MSG for 1 h is sufficient to impair the pulsing and swimming motility of ephyrae as compared with same-time ASW controls. We therefore designed a series of tests in which the ephyrae were treated for 1 h in 5 mM MSG in the presence of either gold chloride (0.022 μM) or D-methionine (5 mM), followed by transfer of the ephyrae to either ASW or ASW with gold, as noted in Tables 1 and 2.

Gold chloride

Ephyrae pre-treated for 1 h with gold chloride before exposure to MSG plus gold chloride for 1 h showed improvement in pulse numbers/min as compared with ephyrae from MSG treatment alone at 3, 24, and 48 h after transfer from the MSG into ASW or ASW + gold chloride. Without gold preteatment, administration of gold chloride simultaneously with MSG generally provided no significant protection from pulsing reduction, although a trend was present and was manifested at 48 h in the case of the gold + MSG into ASW group (see Table 1). Only the gold pretreatment groups eventually recovered pulsing to the point of not being statistically different from the ASW control group.

The number of ephyrae that could swim after receiving gold chloride pretreatment for 1 h prior to MSG treatment as well as groups receiving gold simultaneously with MSG for 1 h was significantly higher than the MSG alone group at periods of 3 and 24 h or 24 h post-MSG treatment, indicating that the gold chloride alleviated the swimming impairment caused by MSG in some ephyrae. The number of ephyrae swimming in all the MSG-treated gold groups was significantly lower than ASW controls, except those at the 24 h time period that had been given gold chloride prior to and simultaneously with MSG and transferred to ASW + gold chloride.

D-Methionine

Ephyrae given D-methionine simultaneously with MSG for 1 h had significantly higher pulse numbers/min than ephyrae given MSG alone at 3, 24,

Table 1. Effects of antioxidants and MSG on ephyrae pulsing rates (pulses per minute)

	Base	15 min	30 min	45 min	60 min	1 h out	3 h out	24 h out	48 h out
MSG									
Mean	107.0	61.7*	68.5*	92.3	46.0*	23.7*	16.2*	13.2*	9.6*
Std dev	46.0	33.6	39.9	36.0	41.7	26.7	13.5	35.0	20.3
n	46	22	11	11	35	32	24	24	18
ASW									
Mean	96.2	**90.4**	**108.5**	104.9	**97.4**	**94.4**	**79.3**	**79.9**	**86.5**
Std dev	40.4	36.1	47.3	44.9	34.8	36.2	31.6	29.8	42.5
n	60	24	13	13	49	44	36	36	24
Met									
Mean	95.3				**115.8**	**89.4**	**104.6***	**79.4**	**74.8**
Std dev	33.8				21.3	29.2	27.3	32.0	33.4
n	12	n.d.	n.d.	n.d.	12	12	12	12	12
MSG + Met									
Mean	97.2				**17.5***	**6.8***	**41.0***	**63.0**	**82.3**
Std dev	23.0				8.6	4.9	36.1	52.6	39.7
n	12	n.d.	n.d.	n.d.	12	12	12	12	12
PreAu/MSG + Au⇒Au									
Mean	92.2				49.9*	19.1*	**29.6***	**64**	**51.2**
Std dev	38.6				45.7	14.8	30.6	54.9	58.4
n	12	n.d.	n.d.	n.d.	12	12	12	12	6
PreAu/MSG + Au⇒ASW									
Mean	102.5				53.9*	23.8*	**30.8***	**46.7***	**65.0**
Std dev	42.9				56.8	28.0	28.5	55.4	61.5
n	12	n.d.	n.d.	n.d.	12	12	12	12	6
MSG + Au⇒Au									
Mean	115.2				39.3*	15.3*	28.3*	30.8*	30.2*
Std dev	50.1				44.4	12.4	31.3	29.4	38.7
n	12	n.d.	n.d.	n.d.	12	11	12	12	6
MSG + Au⇒ASW									
Mean	98.5				41.0*	42.7*	19.0*	20.3*	**41.0***
Std dev	45.5				36.9	55.4	18.0	17.2	36.9
n	12	n.d.	n.d.	n.d.	12	12	12	12	12

* Different from corresponding ASW value, $p < 0.05$.
Bold different from corresponding MSG value, $p < 0.05$.
n.d. – no data.
MSG = 5 mM MSG exposure for 1 h, agents added as noted; ASW = artifical seawater; Met = 5 mM D-methionine (1 h); MSG + Met = 5 mM MSG and 5 mM D-methionine (1 h); PreAu/MSG + Au/Au = 1 h pretreatment with 0.022 μM gold chloride, followed by gold + MSG (1 h), then placed in gold chloride; PreAu/MSG + Au/Au = pretreatment with gold chloride, followed by gold + MSG (1 h), then placed in ASW; Au + MSG/Au = gold chloride + MSG (1 h) then placed in gold chloride; Au + MSG/ASW = gold chloride and MSG (1h) then placed in ASW.

and 48 h post-MSG, but were significantly less active at the 1 h in and 1 h post-MSG time periods. A significantly higher number of the D-methionine plus MSG treated ephyrae swam as compared with those receiving glutamate alone in the 3, 24, and 48 h post-MSG time periods. Ephyrae given D-methionine alone for 1 h maintained pulsing levels equivalent or better than ASW controls throughout the experiment. As was shown also with the gold chloride studies, MSG when given alone caused a statistically significant reduction in the number of ephyrae able to swim and to pulse as compared with ASW controls at all but the 45 min pulsing measurement.

Table 2. Effects of antioxidants and MSG on ephyrae swimming

	Base	15 min	30 min	45 min	60 min	1 h out	3 h out	24 h out	48 h out
MSG swimmers/total	46/47	15/23*	2/11*	2/11*	2/35*	1/32*	1/24*	1/24*	2/18*
ASW swimmers/total	59/59	**23/23**	**13/13**	**12/13**	**49/49**	**44/44**	**46/46**	**36/36**	**24/24**
Met swimmers/total	13/13	n.d.	n.d.	n.d.	**13/13**	**13/13**	**13/13**	**13/13**	**13/13**
MSG + Met swimmers/total	12/12	n.d.	n.d.	n.d.	4/12*	0/12*	**7/12***	**8/12**	**12/12**
PreAu/MSG + Au⇒Au swimmers/total	12/12	n.d.	n.d.	n.d.	6/12*	3/12*	**7/12***	**8/12**	2/6*
PreAu/MSG + Au⇒ASW swimmers/total	12/12	n.d.	n.d.	n.d.	4/12*	3/12*	**6/12***	**6/12***	3/6
MSG + Au⇒Au swimmers/total	12/12	n.d.	n.d.	n.d.	1/12*	0/12*	3/12*	**7/12***	3/6
MSG + Au⇒ASW swimmers/total	12/12	n.d.	n.d.	n.d.	1/12*	0/12*	1/12*	**5/12***	1/6*

* Different from corresponding ASW value, $p < 0.05$.
Bold different from corresponding MSG value, $p < 0.05$.
n.d. – no data.
MSG = 5 mM MSG exposure for 1 h, agents added as noted; ASW = artifical seawater; Met = 5 mM D-methionine (1 h); MSG + Met = 5 mM MSG and 5 mM D-methionine (1 h); PreAu/MSG + Au/Au = 1 h pretreatment with 0.022 μM gold chloride, followed by gold + MSG (1 h), then placed in gold chloride; PreAu/MSG + Au/Au = pretreatment with gold chloride, followed by gold + MSG (1 h), then placed in ASW; Au + MSG/AU = gold chloride + MSG (1 h) then placed in gold chloride; Au + MSG/ASW = gold chloride and MSG (1 h) then placed in ASW.

Glutamate short-term exposure

Tests were done to determine the early effects of MSG on ephyra swimming and pulsing. Ephyrae were given 5 mM MSG and observed every 15 min up to 1 h and 1 h post-treatment for pulsing and swimming effects as compared with ASW controls. We found that swimming is impaired in a significant number of ephyrae within 15 min of MSG treatment and the impairment persists to 48 h post-MSG administration. Pulsing numbers were significantly lower as compared with ASW controls at 15, 30, 60 min, and 1 h post-MSG. The maintenance of pulsing at 45 min may reflect a neurotoxic stimulation of the ephyrae's pacemaker prior to the onset of glutamate toxicity.

Fluorometric studies

Fluorometric studies of calcium and ROS in ephyrae revealed an initial increase in calcium and especially ROS in the ephyrae, with a specific ROS increase in the rhopalia (Fig. 1A and B). In Fig. 1C and D, the presence of baseline NO in the rhopalia is demonstrated. At higher magnification, over 30–40 min post-MSG, the area and intensity of the calcium signal within cells of the rhopalia appear to be increasing (Fig. 1E and F) compared to control cells (Fig. 1C and E). Specific increases in NO, as well as ROS, also occurred in some rhopalia (Fig. 1D) as determined by DAF-FM fluorescence. A plot of the rhopalia rhod-2 and carboxy-H2DCFDA fluorescence confirms statistically significant increases in intracellular calcium and ROS activity over time following 5 mM MSG treatment (Fig. 2).

Discussion

Using *Aurelia* ephyrae, we have shown that extraneously administered glutamate causes a decreased pulsing rate and inhibition of swimming. Swimming cessation and pulsing reduction occur as early as 15 min after administration of the glutamate in many ephyrae. It is not known whether glutamate functions as a neurotransmitter in *Aurelia,* but the effect of glutamate on ephyra swimming strongly suggests that glutamate could have neurotransmitter functions, especially in neurons of the neuromuscular system and pacemakers. Further, glutamate could be affecting ephyra hair cell synapses.

The glutamate results also suggest that ephyrae have specific glutamate receptors which may be found also in other coelenterates. In *Hydra*, Kass-Simon & Scappaticci (2004) found that glutamate causes a significant increase in the number of

stenoteles (nematocysts) responding to direct mechanical stimuli. The effect was mimicked by NMDA together with kainate or NMDA with AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid). These authors concluded that a glutamatergic mechanism working through iono-

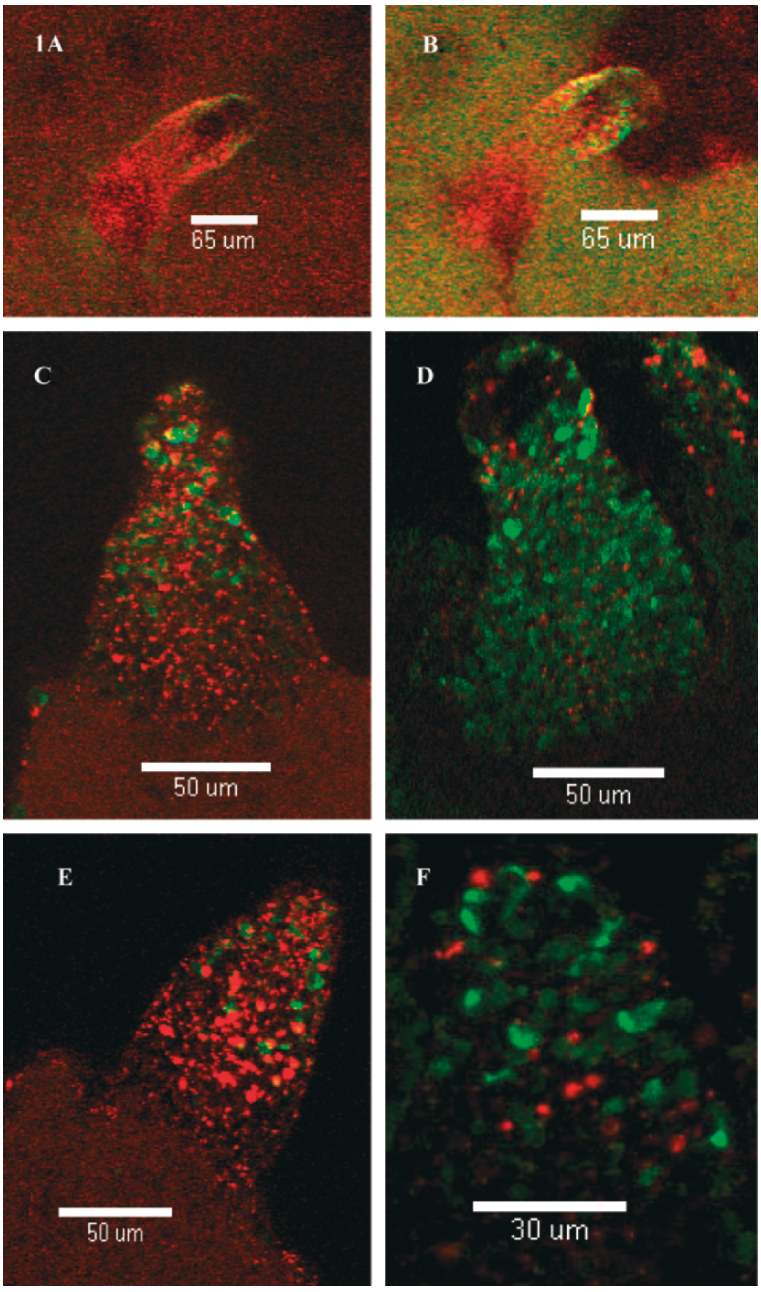

Figure 1. Note that due to movement of the ephyrae, there were changes in the orientation of the lappets and rhopalia between measurements. (A) Control rhopalium with rhod-2 AM and carboxy-H2DCFDA, seen with 10× objective. (B) Same rhopalium as A after 15 min exposure to 5 mm MSG. Note increase in ROS fluorescence in rhopalium and in surrounding lappet. Measurements also confirmed increase in rhod-2 signals in rhopalium. (C and D) Control rhopalia of two ephyrae loaded with rhod-2 and DAF-FM diacetate, seen with 25× objective. (E) 30 min after 5 mM MSG and seen with the 25× objective, the rhopalium in C shows increased calcium fluorescence. (F) 32 min after exposure to 5 mm MSG and seen with the 40× objective, the rhopalium of D shows increases in calcium and NO.

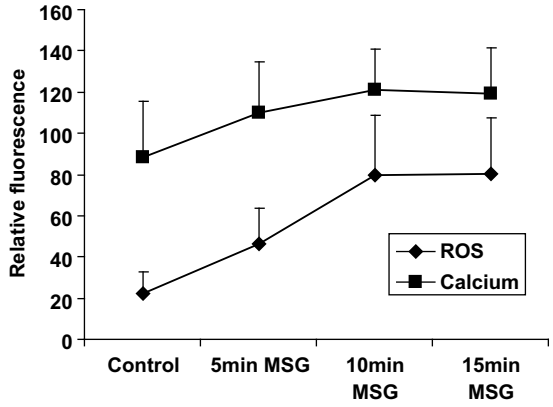

Figure 2. Elevation of intracellular calcium and ROS in ephyrae treated with 5 mM MSG. Ephyrae were loaded with rhod-2 AM and carboxy-H2DCFDA, treated with 5 mm MSG, and their fluorescence determined as described in the Methods section. Results of seven measurements were combined and analyzed by paired *t*-test, with the 5, 10, and 15 min time points found statistically significantly increased ($p < 0.05$) from time 0 values.

tropic glutamate receptors appears to lower the firing threshold of stenoteles (which are innervated), perhaps by permitting the entry of Ca^{2+} into the cells through the early evolved NMDA/kainate/AMPA mechanism. Bellis et al. (1991) had identified two distinct binding sites for L-glutamic acid in a crude membrane fraction from *Hydra vulgaris*. Also, Smith et al. (2004) found and cloned a glutamine synthetase gene from the sea anemone *Aiptasia pallida*.

In mammals, glutamate has neurotransmitter functions. Glutamate excitotoxicity, which occurs as a result of excessive glutamate, has been described as causing increasing damage of cell components, including mitochondria, leading to cell death. In the process, ROS are generated (Atlante et al., 2001). During the course of the cell damage, increases in O_2^- and hydrogen peroxide (H_2O_2) occur (Ishikawa et al., 1999). These authors reported that both H_2O_2 and O_2^- play important roles in the resultant apoptosis in embryonic hippocampal neurons in culture. Additionally, NO and/or peroxynitrite are involved in the cell destruction.

Fluorometric studies of calcium, ROS, and NOS in ephyrae revealed increased calcium-, ROS-, and NOS-generated nitric oxide (NO) in the ephyrae as early as 5 min, with increases up to 50 min after glutamate treatment (data not shown). This result suggests that swimming impairment is associated with the calcium influx and ROS increases, which are early events in glutamate excitotoxicity in mammals.

A number of antioxidants reduce glutamate excitotoxicity effects (Miyamoto et al., 1989; Mazzio et al., 2001), including D-methionine and gold compounds. In *Aurelia*, ephyrae given D-methionine simultaneously with MSG for 1 h had significantly higher pulse numbers/min than ephyrae given MSG alone at 3, 24, and 48 h post-MSG. More ephyrae, treated simultaneously with D-methionine and MSG for 1 h, were able to swim at all times from 3 h post-glutamate treatment as compared with those receiving MSG alone. D-methionine protects against cisplatin-induced hearing loss when co-administered or administered prior to cisplatin in the Wistar rat (Campbell et al., 1996; Reser et al., 1999). According to Smoorenburg et al. (1999), D-methionine, which is a thiol compound, may protect against cis-platin-induced hearing loss through the scavenging of cisplatin-induced free radicals. D-methionine also protected against gentamicin-induced free radical generation in a non-enzymatic system *in vitro* and in the guinea pig *in vivo*, according to Sha & Schacht (2000).

Gold chloride also may function as an antioxidant in *Aurelia* ephyrae. Gold-chloride-treated ephyrae pre-treated for 1 h before exposure to MSG plus gold chloride showed improvement in pulsing as compared with ephyrae from MSG treatment alone at 3, 24, and 48 h after transfer from the MSG into ASW or ASW + gold chloride. The number of ephyrae that could swim after receiving gold chloride for 1 h prior to MSG treatment as well as simultaneously with MSG was significantly higher than the number of ephyrae swimming after treatment with MSG alone at 3 and 24 h post-MSG treatment, indicating that the gold chloride alleviated the swimming impairment caused by MSG in some ephyrae. Improvement in pulsing and swimming ability may be directly associated with recovery of motor and/or pacemaker neurons damaged by MSG. Richards et. al. (2002) recognized the need for more research of the neuropharmacology and neurochemistry of gold.

Many therapeutic gold-containing drugs are gold (I) compounds whereas gold chloride is a gold

(III) compound. Gold (I) drugs selectively activate the DNA binding of a heterodimer consisting of the basic-leucine zipper transcription factors Nrf2 and Maf (Kataoka et al., 2001). Once bound to its recognition DNA sequence termed antioxidant-responsive element, Nrf1/Maf induces a set of antioxidant stress genes whose products contribute to the scavenging of ROS and exhibit anti-inflammatory effects. Graham et al. (1993) noted that aurocyanide, which is a potent inhibitor of the oxidative burst of neutrophils, may be metabolized to Au(III) complexes.

Other gold drug studies emphasized the use of activated white blood cells, which produce superoxide ions when activated, to determine effectiveness of gold drugs in reducing the levels of these O_2^- ions. Auranofin (Hafstrom et al., 1984) and aurothiomalate and triethylphosphine (Davis et al., 1983) are among gold compounds effective in inhibiting superoxide radical production. More recently, Aaseth et al. (1998) noted that therapeutically given gold salts, through a lysosomal loading of the metal, reduce toxic oxygen production.

Klein & Ackerman (2003) stated that the mechanisms by which neurons die under conditions of oxidative stress remain largely unknown. These authors point out that excessive ROS can lead to the destruction of cellular components including lipids, protein, and DNA, and ultimately cell death is via apoptosis or necrosis. Glutamate neurotoxicity has been linked to mitochondrial uptake of Ca^{2+} as well as to the activation of calmodulin and neuronal nitric oxide synthase in motor neurons (Urushitani et al., 2001). Fluorometric analysis showed that mitochondrial Ca^{2+} was elevated promptly following exposure to 0.5 mM glutamate for 5 min with subsequent accumulation of ROS in the mitochondria. The NMDA receptor was also activated. Fluorometric analyses of free radicals in ephyrae following treatment with 5 mM MSG indicate an increase of free radicals, including NO, in the ephyrae as early as 5 min following treatment. Vergun et al. (2001), using inhibitors of lipid peroxidation and scavengers of superoxide or hydrogen peroxide during and following a 10 min exposure to glutamate, found that the antioxidants delayed but did not prevent the glutamate-induced mitochrondrial depolarization and the secondary Ca^{2+} rise, but they did exert protective effects against glutamate-induced neuronal death at 24 h following a 10 min excitotoxic event. This protective effect against glutamate-induced neuronal death apparently occurred through steps downstream of a sustained increase in Ca^{2+} associated with the collapse of mitochondria potential.

Conclusion

Glutamate (MSG) causes motility abnormalities including low pulse numbers and swimming cessation in *Aurelia* ephyrae. Swimming and pulsing are impaired in a significant number of ephyrae within 15 min of MSG treatment. The mechanism of MSG actions on ephyra motility is unknown, but glutamate is known to damage neurons and hair cells of higher animals possible through the formation of ROS. Microscopic fluorometric analyses of calcium and free radicals, including NO, indicated an increase of these substances in the ephyrae as early as 5 min following MSG treatment. Tests using D-methionine and gold chloride, known antioxidants, revealed that both compounds accelerated the time of recovery from MSG-induced motility impairment, possibly through their antioxidant activities.

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Medusoid release and spawning of *Macrorynchia philippina* Kirchenpauer, 1872 (Cnidaria, Hydrozoa, Aglaopheniidae)

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Key words: life cycle, sexual reproduction, hydroids, Indian Ocean

Abstract

The life cycle of the aglaopheniid *Macrorynchia philippina* Kirchenpauer, 1872, is re-described from examination of live specimens collected from Réunion Island, Indian Ocean. Fertile colonies were collected on the outer slope of the coral reef and medusoid release happened a few hours later. Video sequences were recorded. Colonies were hermaphroditic: each phylactocarp contained one female and one male gonotheca. Sexual dimorphism was remarkable: sex could be recognized by colour, the female being red ochre, including about 40 oocytes disposed in a mosaic feature, and the male yellow ochre, having a homogeneous mass of spermatozoa. The blastostyle ran all around the gonangium near the closure of the two valves of the gonotheca, forming gubernacula. A ring of refringent corpuscles was clearly visible near the apex. Medusoids were indistinguishable inside the gonotheca. Male and female medusoids were released simultaneously at gamete maturity. Medusoid release involved the basal rupture of the blastostyle and the rupture of the links between the ectoderm surrounded the medusoid (the mantle), including the blastostyle (the mantle), and the gonothecal perisarc. While the two valves of the gonotheca were pushed and drew aside, the medusoid emerged by slipping out of the mantle that ruptured distally, forming a sheath; the bell of the medusoid did not contract. Immediately after emergence, quick and strong contractions of the bell allowed the medusoid to swim and induced spawning by breaking the ectoderm surrounding the gametic mass around the spadix. Spawning lasted only a few minutes: both oocytes and spermatozoa were expelled at each contraction. Spent medusoids remained alive only about 2 h. External fertilization gave rise to planulae 1 day later.

Introduction

In the suborder Plumulariida, which comprises families Syntheciidae, Sertulariidae, Kirchenpaueriidae, Halopteriddidae, Plumulariidae, and Aglaopheniidae, production of gametes is mainly ensured by the benthic stage, i.e. inside fixed gonophores on the polyp (Bouillon, 1985; Cornelius, 1992), the planulae being brooded inside the female gonotheca or in specialized structures developing at its proximity. It is generally assumed that medusae have disappeared through paedomorphic processes (Boero et al., 1997). However, some studies indicate the production of free medusoids (Motz-Kossowska, 1907; Teissier, 1926; Gravier, 1970; Migotto, 1996, 1998; Migotto & Marquès, 1999; Gravier-Bonnet & Migotto, 2000; Gravier-Bonnet & Lebon, 2002). Although differences exist among medusoids already described, they have common features. They are released by the colony when gametes are ripe, being short-lived structures devoted to gamete dispersal. A medusoid lacks tentacles and bulbs, circular and radial canals, and mouth and sense organs, but the

umbrella is bell-shaped, with an exumbrella, a subumbrella, and a velum, as for a fully-formed medusa. In place of a manubrium, it has an eccentric spadix. The bell can pulsate so the animal can swim and spawn. Both male and female gametes are expelled during swimming and thus fertilization occurs in the water. Spent medusoids die quickly. These medusoids are cryptomedusoids, as described in 1926 by Teissier, who classified free medusoids as including eumedusoids, cryptomedusoids, and heteromedusoids.

Macrorynchia philippina Kirchenpauer, 1872, is a circumtropical species (Millard, 1975) and the first free medusoid-producing species known among Aglaopheniidae. Gravier (1970) first described the liberation of free medusoids from epibiotic specimens on the marine phanerogam *Thalassodendron ciliatum* (Forskal) in coral reefs of Madagascar. Cornelius (1990) and Calder (1997) questioned these results as Vervoort (1968) and Hirohito (1983) had described, respectively, a single egg inside gonothecae of specimens from Venezuela and one to three eggs or planulae inside gonothecae of specimens from Japan. On the coral reefs of La Réunion, a tropical oceanic island in the Indian Ocean (21° 50′ S, 55° 30′ E), *M. philippina* was previously found by Gravier-Bonnet (unpublished data), but only small sterile specimens had been collected until now. Finding a large fertile colony from the same area provided the opportunity to observe and to record the release and spawning of male and female medusoids. We corroborate previous results from Madagascar (Gravier, 1970).

Materials and methods

Live material was collected by scuba diving on the outer slope of the fringing reefs located on the western coast of La Réunion. The macroscopic colonies were collected by hand. Collection was part of a wider study on Plumulariida reproduction done from February to May 2002 (from the end of the austral summer to the autumn, water temperature 29–26 °C). Fertile specimens were sampled on 9 March 2002, at 21 m, at 'Tour de Boucan'. Methods used for collection, transportation, and observation of specimens were similar to those described in Gravier-Bonnet & Lebon (2002). Live samples were kept 1 day in a closed system aquarium where medusoid release was observed. Male and female medusoids were sampled just after release and isolated in a Petri dish. To separate the corpuscles from the tissues, in order to study their chemical composition, spent medusoids were desiccated, then rehydrated with hydrogen peroxide (P. Bachèlery & P. Frouin, personal communication). Identification of the species and observation of the reproductive structures and processes were made under a stereomicroscope (Leica M420 and M32) and with a photo microscope (Leitz Diaplan). Video sequences were taken with a mono CCD camera (Sony) connected to a video recorder and to a computer. Still photographs were grabbed from the video, and plates were prepared using Adobe Photoshop.

Results

Fertile colony and gonangium morphology

The colony was 150 mm high with a typical white-blue iridescent colour and fascicled stem and branches. It stung humans, as usual for this species in other locations, due to the presence of many large microbasic mastigophores (fired nematocyst capsules 67–69 × 5–6.5 μm; shaft 66–73 μm long). The colony bore both male and female gonothecae on modified hydrocladia called phylactocarps (usually each had two hydrocladia, one modified as a phylactocarp; Fig. 1a); phylactocarps were located on secondary branches. About 20 gonothecae were counted on a single branch, and 10 branches were provided with gonothecae. Each phylactocarp bore at its base a normal hydrothecal article and two gonothecae, a female proximal and a male distal (Fig. 1b), and ended with a long curved portion formed by several articles with long nematothecae that ensured its protection. Gonothecae were round in shape and strongly flattened laterally; they were formed of two symmetric valves (Fig. 1c and d). The sex of gonads was recognizable through the transparent perisarc. Inside a female, about 40 clearly defined oocytes, with dark nuclei, were disposed as a mosaic (Fig. 1g and h). A male showed a homogeneous mass of spermatozoa (Fig. 1e and f). Female col-

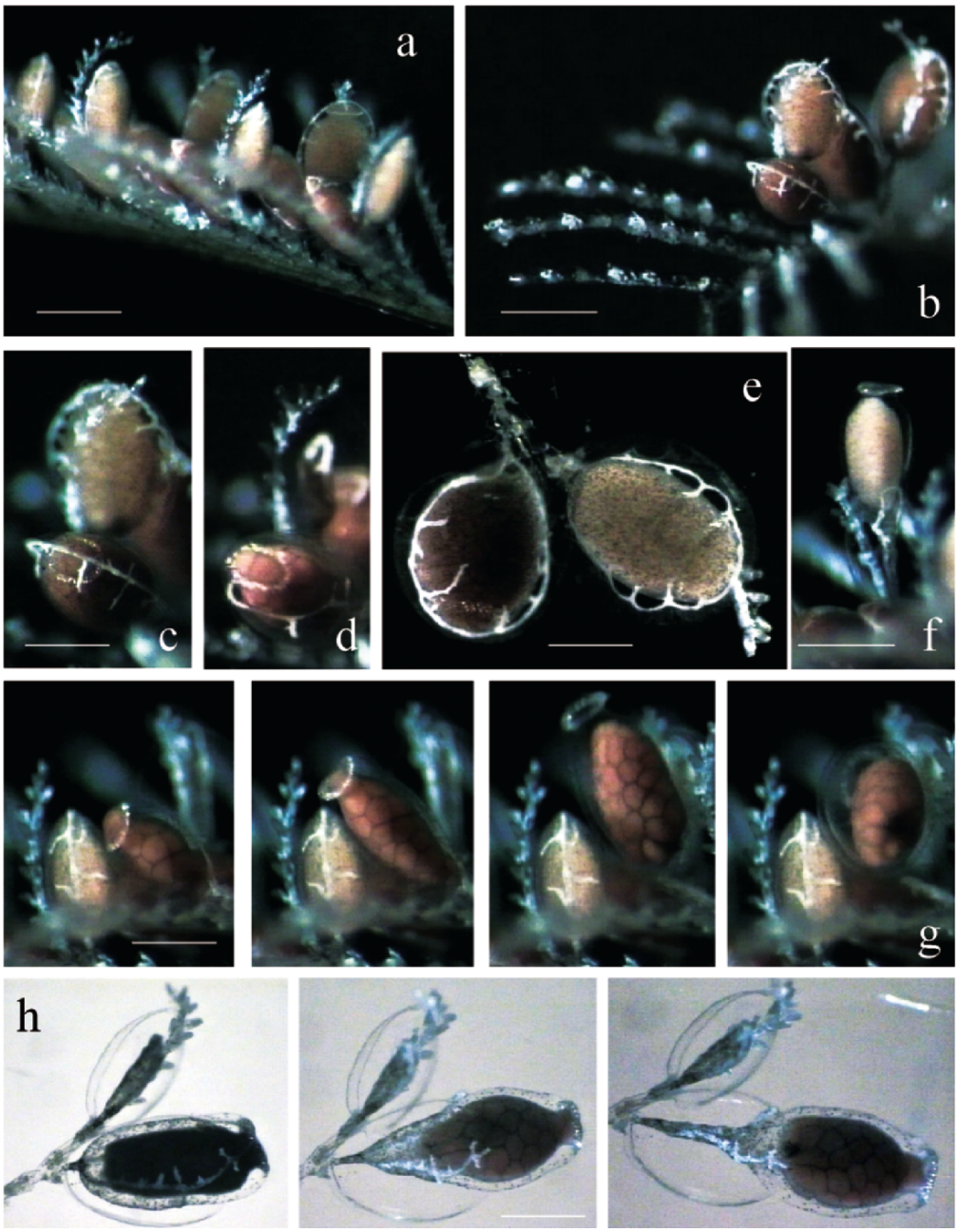

Figure 1. *Macrorhynchia philippina*: medusoid release. (a) Lateral view of a branch with phylactocarps bearing male and female gonangia. (b) View from above of two phylactocarps with proximal female and distal male gonothecae. (c and d) Details before and during female medusoid release. (c) With a male medusoid still inside the gonotheca, and (d) lacking male medusoid, which was liberated before the female medusoid; notice the refringent corpuscles. (e) Gonothecae where the ectoderm detaches from the perisarc just before medusoid release, and with blastostyle plus gubernacula. (f) Profile view of male medusoid issued from the gonotheca, with the remaining ectodermal sheath bearing blastostyle and gubernacula on one side. (g) Four steps of female medusoid release, from left: engaged, with oocyte mass compressed and extruding through the ring of corpuscles; still engaged; completely free with the bell and the oocyte mass rightly positioned; first contraction inducing the swimming by the apex of the bell. (h) Three steps of the slipping out from both the gonotheca and ectodermal sheath of a female medusoid (under an empty male gonotheca). Scale bars a–b, e–f, 1 mm; c–d, g–h, 0.75 mm. All photographs are from video records of living material; a–g, episcopy; h, diascopy and episcopy.

our was red ochre and male yellow ochre. The gametes were densely packed and filled the gonotheca. The blastostyle, a dense white line, ran around the gonad, near the closure of the two valves of the gonotheca, and was divided laterally into gubernacula (Fig. 1e). A thin ectodermical epithelium (the mantle) enveloped the blastostyle. It was located underneath the skeleton, was linked to it, and was quite invisible, except for the presence of pigmented cells. A ring of refringent corpuscles, perpendicular to the main axis of the gonotheca in males and females, was visible near the apex of the gonadic mass (under the blastostyle when the valves were closed) (Fig. 1a–h).

Medusoid release

The release of medusoids occurred at dusk for colonies collected on the morning. Medusoids emerged by slipping between the two valves of the gonotheca, which drew aside. It lasted about 3 min in lab conditions. First, the ectodermical epithelium detached from the gonotheca (i.e. the links were broken, Fig. 1e) and then the medusoid began to slide out, base first, without any contraction of the bell. From just before the opening of the gonotheca and during the slipping of the medusoid, the gametogenetic mass protruded through the velum and above the ring of corpuscules, by contrast with its previous position (Fig. 1a–e). Some oocytes could even pass through the aperture of the velum but as they were surrounded by the ectodermical epithelium covering the gonad, they recovered their initial position when the medusoid was no longer squeezed (Fig. 1d and g). By drawing aside, the two valves of the gonotheca created a narrow passage and, moreover, the ectoderm (the mantle) enclosing the blastostyle opened, forming a sheath that compressed the medusoid that glided out (Fig. 1f and h). The ectoderm was broken but not the blastostyle and its gubernacula, visible entirely on one side of the sheath (Fig. 1d and f). Medusoids seemed to slide like a cake of soap in your hand in a bath, but very slowly. Once the medusoid was outside but still linked, strong contractions of the bell occurred immediately. For a few seconds, the medusoid remained connected to the colony, but the short strong contractions ended with its liberation into the water. The release left a conical scar at the top of the medusoid (Fig. 2c and d). Inside the empty gonotheca, the mantle shrank toward the base (Fig. 1h).

Medusoid morphology

The bell of a free medusoid (Fig. 2a–e) was taller than wide, and was formed by an exumbrella and a subumbrella separated by a thin layer of mesoglea that was thickened at its base over the circle of corpuscles. Inside the subumbrellar cavity, the mass of gametes was still densely packed, completely covering the spadix. The refringent corpuscles formed a ring near the margin between the bell and the velum (Fig. 2g). Of different size, they were disposed in two or three layers. When exposed to hydrogen peroxide after being desiccated, a concentric structure (Fig. 2g insert) was revealed. The amount of material was not sufficient to determine the chemical composition of refringent corpuscles. The exumbrella contained many pigmented cells and scattered small microbasic mastigophores; the subumbrella and the inner side of the velum had muscular fibres clearly visible under the microscope. The ectodermal side of the velum was provided with pigmented cells too. A medusoid lacked circular and radial canals, tentacles and bulbs, and sensory organs like ocelli or statocysts. When empty, the spadix became visible and looked slightly eccentric in lateral view (Fig. 2f, left). There was no mouth.

Spawning

The release of male and female medusoids, and therefore the shedding of the gametes, was simultaneous. Unspent medusoids swam very quickly. Spawning was accomplished during swimming and began 10 s or so after release, lasting 1–2 min. Just after the release, contractions of the bell induced not only swimming but also the breaking of the ectoderm that enveloped the gametes that were densely packed around the spadix. This led the oocytes to begin to detach from the spadix and to separate from one another, while their nuclei became invisible (Fig. 2e). Oocytes became free inside the subumbrellar cavity before being expelled but they were still polygonal when spawned and became round only later. The strong, very short contractions were grouped in long series separated

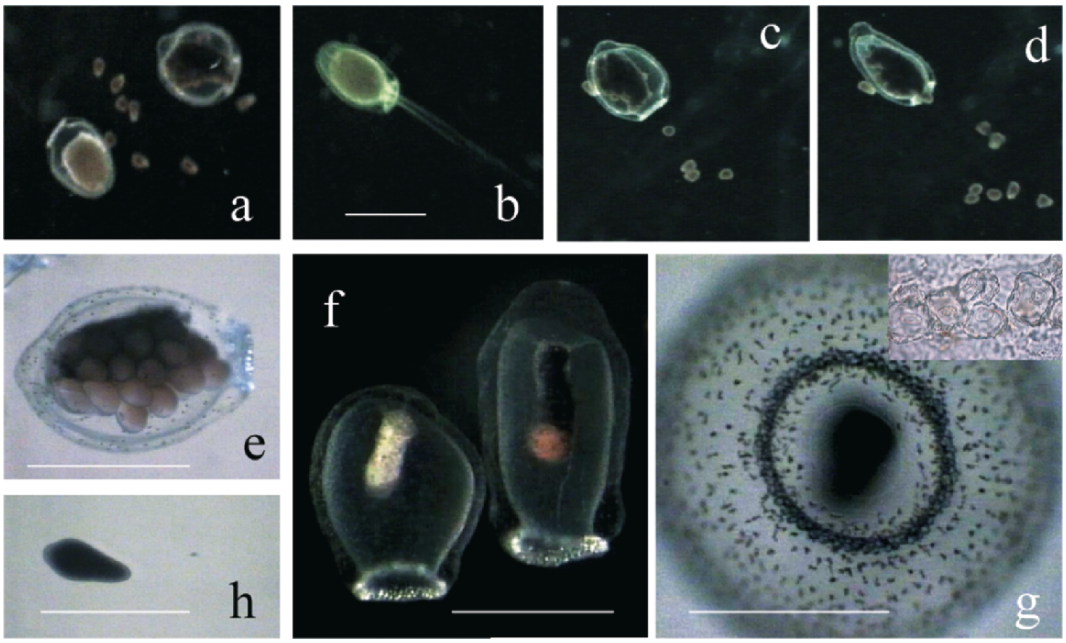

Figure 2. Macrorhynchia philippina: medusoid structure and spawning. (a) Sexual dimorphism of male and female medusoids during spawning. (b) Male expelling spermatozoa during swimming by contraction of the bell. (c and d) Female spawning: a few oocytes expelled during a single contraction of the bell. (e) Female with oocytes detaching from the spadix and mesoglea thickened around the orifice of the bell. (f) Dead empty medusoids with eccentric spadix, white in male and black in female where one oocyte remains stuck. (g) Empty live female medusoid from below, showing bell orifice with velum encircled by a ring of corpuscules, and pigmented cells on the exumbrella and on the velum; insert shows concentrically-arrayed refringent corpuscles after medusoids were desiccated and exposed to hydrogen peroxide. (h) Planula. Scale bars a–f, 1 mm; g–h, 0.5 mm. All photographs are from video records; a–d, f, episcopy; e, diascopy plus episcopy; g, h, diascopy.

by phases of rest. Male medusoids swam, leaving a trail of white spermatozoa behind, and females released unfertilized eggs, 1–4 at each contraction (Fig. 2b–d). Spermatozoa diluted in the water and eggs sank to the bottom of the Petri dishes.

Spent medusoid and planulae

Spent medusoids (Fig. 2f and g) remained alive for about 2 h, during which the frequency of contraction series diminished; then they died. Sexual dimorphism was still visible mainly because of the shape of spent medusoids and the colour of the spadix: a female medusoid was slightly taller than a male and had a black spadix while a male had a beige one.

Self-fertilization occurred in the water. Segmentation began rapidly and planulae were formed within 24 h. They were very short and polarised, with a large and globular anterior and a slender posterior (Fig. 2h). They swam and crawled in the dishes. Settlement was not observed.

Discussion

These results from Réunion Island corroborate data from Madagascar (Gravier, 1970). *Macrorynchia philippina* from the Indian Ocean is a hermaphrodite that produces both male and female free medusoids (cryptomedusoids), so is a broadcast spawner. These results agree with data from Brazil (Migotto, 1996). In hydroids, reproductive events can be influenced by environmental conditions (Boero & Sarà, 1987; Gili & Hughes, 1995). A species may reproduce in different ways depending on temperature, depth, or latitude, which could explain the observations of Vervoort (1968) and Hirohito (1983) cited in the Introduction. Other species like *Orthopyxis integra*, *Sarsia tubulosa*, and *Halocordyle disticha* can liberate

short-lived eumedusoids or retain them on the colony as fixed gonophores (Agassiz, 1862; Stefani, 1959; Brinckmann-Voss, 1970). This phenomenon was called allogony by Giard (1898). If this is true for the specimens from Venezuela and Japan, it is probably a matter of taxonomical identification. The specimens originating from Japan (Hirohito, 1983, 1995) differ from *M. philippina* in having (1) erect lateral nematothecae extending above the level of the orifice of the hydrotheca instead of ending at the orifice level and lying against the hydrothecal wall, (2) an internal hydrothecal septum linear and not triangular (in lateral view), the abcauline wall being thin instead of thickened, and (3) phylactocarps with more than two gonothecae. In addition, the general shape of the hydrotheca and hydrothecal article are very similar. Within the Plumulariida, as in other large taxa, there are numerous sympatric species that have not been verified and could be distinguished only by new studies including observation of new characters including molecular data (Gravier-Bonnet, personal observations). Such research had permitted separating three species on Réunion Island that were previously all considered to be *Sertularella diaphana* (Allman, 1885) (Gravier-Bonnet, unpublished data).

The medusoids from Madagascar (Gravier, 1970) and these from Réunion Island are similar but differ in a few characters. First, female medusoids from La Réunion had about 40 oocytes instead of about 80 for those from Madagascar, a difference that could be related to water temperature and season and/or size of the colonies. The colony from Madagascar, which was 200 mm high, was collected on 22 August 1969 (therefore in winter), while that from La Réunion, which was 150 mm high, was collected on 9 March 2002 (so in summer). In an athecate species (*Rhizogeton* sp.), such a discrepancy in number of eggs was related to season (Gravier-Bonnet, unpublished data). Second, the period of medusoid release (in lab conditions) was at dusk in Réunion Island and in the morning in Madagascar. However, the stress of sampling might have induced spawning since gametes were already mature. In both, medusoid release occurred within 24 h of collection, spawning and fertilization occurred in the water, and planulae were formed and able to swim the following day. Self-fertilization, followed by healthy planula development, was obtained in lab conditions. In both cases, sexual dimorphism of the gonangia is remarkable. Male and female medusoids, either unspent or spent, could be recognized by colour: males were clearer than females.

As already noted, free cryptomedusoids (sensu Teissier, 1926) in the thecate life cycle may be more widespread than supposed (Boero & Bouillon, 1989). Studies of sexual reproduction remain rather rare. Well-documented data on cryptomedusoids liberated by the polyp stage come from the following species: *Amphisbetia operculata* (Linneus, 1758); *Sertularella diaphana* (Allman, 1885) and *Sertularella* sp.; *Sertularia marginata* Kirchenpauer, 1864, *Sertularia loculosa* Busk, 1852 and *Sertularia turbinata* (Lamouroux, 1816); *Monotheca obliqua* (Johnston, 1847); *Dentitheca bidentata* (Jäderholm, 1920); *Macrorynchia philippina* Kirchenpauer, 1872; *Gymangium ferlusi* (Billard, 1901) and *Gymnangium hians* (Busk, 1852); *Nemalecium lighti* (Hargitt, 1924) (Motz-Kossowska, 1907; Teissier, 1922; Gravier, 1970; Migotto, 1998; Migotto & Marquès, 1999; Gravier-Bonnet & Migotto, 2000; Gravier-Bonnet & Lebon, 2002, respectively). In total, eight species in suborder Plumulariida (four Sertulariidae, two Plumulariidae, and two Aglaopheniidae) and only one in Haleciida.

Mechanisms involved in medusoid release are still unknown, but they do not include medusoid contractions. Gravier (1970) said that the valves moved aside by the internal pressure of the gonophore while the coenosarc retracted to its starting point. The same events were observed here but we also noticed (1) the rupture of links between the epithelial ectoderm (the mantle) and the perisarc, (2) the distal opening of the ectodermic sheath around the medusoid, and (3) the concurrent shrinkage of the sheath allowing the medusoid to escape from the gonotheca. Besides biochemical signalling, the increasing volume of the gametes during their maturation could be involved in forcing gonotheca breakage by increasing internal pressure that would also stretch and break the basal junction between medusoid and blastostyle. How the connections between the skeleton and the ectodermal envelope surrounding the medusoid break also remains unknown. The medusoid, once detached from the perisarc, might also be squeezed toward the distal opening of the gonotheca by the

pressure of the shrinking sheath. The valves are amorphous structures like the operculum or opercular valves of the hydrothecae of other species (Hughes, 1992). Therefore, release is a fast process inside the gonotheca, the two valves opening passively under the pressure of the medusoid: the gametic mass protrudes through the velum, becoming visible over the ring of corpuscles (Fig. 1a, second gonotheca from right). For *Nemalecium lighti* (Hargitt, 1924), a species of Haleciidae, development of the gonangium and release of the medusoid were coordinated with the elongation of the blastostyle (Gravier-Bonnet & Migotto, 2000). Moreover, detachment of the mantle that slid along the medusoid was inferred to provide a pressure that induced its slipping and, as a consequence, the aperture of the gonothecal operculum and the exit, as for *M. philippina*.

What is the function of the refringent corpuscles and could they be involved in the liberation process, too? It would be attractive to hypothesize that they could provide positive buoyancy by allowing the medusoids to slip upward. These corpuscles are lacking in Sertulariidae (Teissier, 1922; Migotto, 1998), but, as shown by Gravier-Bonnet & Lebon (2002), medusoids of *Sertularella diaphana* (Allman, 1885) and *Sertularella* sp. slip out in the same way as in *M. philippina*. Therefore this hypothesis is not supported. Moreover, these structures seem to have negative buoyancy, as the spent medusoids of *M. philippina* sank to the bottom of Petri dishes, oral side downward, as observed by Gravier (1970) and Gravier-Bonnet & Migotto (2000), who considered them to function as ballast or statoliths. We do not know the chemical composition of the corpuscles in the medusoids belonging to Plumulariidae, Aglaopheniidae, and Haleciidae. Further investigations will be needed to understand their formation, structure, and function. Additional information is also necessary to compare structure and behaviour of medusoids that have or lack a corpuscle ring.

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Gonad development and somatic growth patterns of *Olindias sambaquiensis* (Limnomedusae, Olindiidae)

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Key words: somatic growth, allometric growth, hydromedusae, *Olindias sambaquiensis*, Blanca bay, Argentina

Abstract

The stinging hydromedusa *Olindias sambaquiensis* (Limnomedusae, Olindiidae) was examined monthly over a 24-month period in Blanca Bay (Buenos Aires, Argentina) in order to determine the relationship between gonad development and overall growth patterns of medusae. *Olindias sambaquiensis* occurred seasonally from November (spring) to late April (fall). Medusae grew until February (mean bell diameter: 76.2 mm ± 2.8), and began to decrease in diameter up to late April (mean bell diameter: 58.6 mm ± 4.2). From November to December, the gonads of medusae were either undifferentiated or not present (gonad index (GI) 4.9%). Sexually mature individuals were observed from January to late February, where GI increased up to 31.4%, reaching, in some cases, values higher than 70%. From late February to April, *O. sambaquiensis* spawn and GI decreased to 25%. Highest growth rates were observed during December (immature phase), and then decreased until February (maturation phase). Growth rates were negative throughout March and April (spent phase). Growth patterns of gonads were different from those of the rest of the medusa: (1) their growth rates (and shrinking rates) were higher than those of the rest of the body, and (2) the percent of 'total organics' (AFDW: ash-free dry weight) in gonads remained constant, while it decreased in the rest of the body. Medusa length-weight relationships were also affected by gonad weight: a positive allometry was observed only when gonad weight was included into the total weight (i.e., whole individual).

Introduction

Hydrozoans and scyphozoans have bipartite life-histories in which asexually reproducing benthic polyps develop into pelagic sexually reproducing medusae (Fautin, 2002). Growth patterns of scyphomedusae and hydromedusae are characterized by three main phases: (1) high growth rates during juvenile phases, when gonads are not present; (2) reduced growth rates at sexual maturity, when gonads develop and reproduction takes place, and (3) reduction of bell diameter and weight after reproduction has occurred (Hamner & Jenssen, 1974; Arai, 1992; Lucas, 1996; Ishii & Båmstedt, 1998). For example, growth rates of scyphomedusae of *Aurelia aurita* decline once development of gonads begins, and their size decreases following reproduction (Möller, 1980; Lucas, 1996, 2001; Ishii & Båmstedt, 1998). Similarly, the growth rates of the hydromedusae *Aequorea victoria* and *Cladonema californicum* are high during juvenile phases, but decline as gonads develop (Arai, 1980; Costello, 1991). The reduction in the size of medusae following reproduction may

be associated with starvation (Frandsen & Riisgård, 1997; Lucas & Lawes, 1998) and/or the onset of genetically determined senescence (Möller, 1980; Lucas, 2001).

Most studies on the growth of medusae have either investigated changes in the chemical composition of gonads and other body parts (Larson, 1986a), or have determined egg production and/or gonad indices over the course of development (Larson, 1986b; Lucas & Lawes, 1998). There have only been two studies that have separated the growth rates of gonads from the growth rates of other features of medusae over the course of maturation, and both of these studies have been made on scyphozoans (Shenker, 1985; Lucas, 1994). There have been no studies examining the relationship between the growth of gonads and other medusae features in hydrozoans.

Olindias sambaquiensis is a hydromedusa that belongs to the family Olindiidae, which has primitive polyps and conspicuous medusae (Nagao, 1973). This species is endemic to the temperate southwestern Atlantic Ocean, and its medusae appear in large numbers in the southern beaches of Buenos Aires every summer (from December to March) (Macchi et al., 1995; Mianzan & Ramírez, 1996). This species is responsible for severe stings to bathers and has adversely affected the tourism industry (Kokelj et al., 1993, 1995; Mianzan et al., 2001). The lack of studies dealing with the relationship between somatic growth and maturation in hydrozoans is an impediment to understanding the population dynamics of these organisms. Therefore, in order to better understand the population dynamics of *O. sambaquiensis* and other hydrozoans, we investigated the relationships among gonad development, reproduction, and the somatic growth of their medusae.

Materials and methods

Medusae of *Olindias sambaquiensis* were sampled monthly over a 2 year period (1982–1984) in Blanca Bay, Buenos Aires, Argentina (Fig. 1). Two plankton samplers were used for the collection of juvenile (Hensen net, 50 cm of mouth diameter, 224 μm-mesh size) and adult (IKMT, 6.25 m^2 of mouth, 0.5 cm-mesh size) individuals, between 1 and 5 m depths. Water temperature (°C) measurements were made at 1 m depths at each station.

All individuals collected ($n = 170$) were washed with distilled water to remove the excess salt. The remaining water on the surface of each specimen was carefully soaked up with blotting paper. Bell diameter (BD) of each individual was then measured in order to establish mean size and size-frequency distributions. Gonads were removed with scissors and the weights of the gonads and the rest of the medusae (i.e., umbrella, tentacles, and manubrium) were determined (see Fig. 2). A subsample of gonad tissue was excised and examined microscopically in order to establish both sex and gonad stage of each specimen (see Macchi et al.,

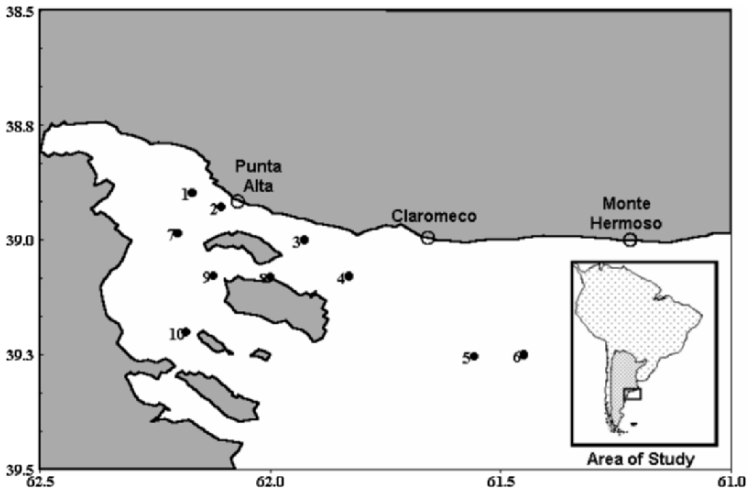

Figure 1. Location of study site and sampling locations in southern Buenos Aires Blanca Bay, Argentina.

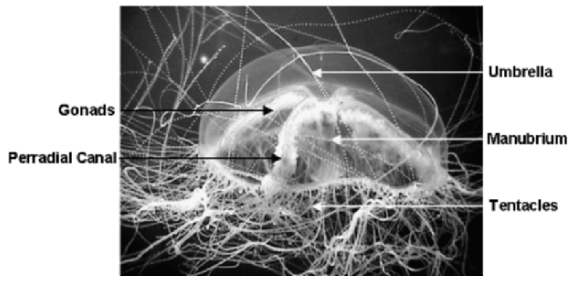

Figure 2. Olindias sambaquiensis (BD 70 mm). Manubrium, tentacles, and umbrella were collectively referred to as the 'Bell'.

1995, for details). Both dry weight, as % wet weight (DW, constant weight at 60 °C) and ash-free dry weight, as % of DW (AFDW, 24 h at 550 °C) of the bell (BDW and BAFDW) and gonad tissue (GDW and GAFDW) were determined. Lucas (1994) showed that the use of AFDW overestimates the amount of 'total organics'. However, and in order to be comparable with previous reports, the term 'total organics' will be used here as meaning AFDW. Gonad Index (GI) was calculated according to the equation:

$$GI = (GW/TW) \times 100,$$

where GW is the GAFDW and TW represents the TAFDW, expressed in grams.

Log-transformations ($Y = \log(X)$) were applied to BD, DW (g), and AFDW (g), for gonads, bell, and the whole individual. Proportional data such as DW (%WW), AFDW (%DW), and GI were arcsine transformed ($Y = \text{arcsine}(X)^{0.5}$) to normalize their distributions.

Data from both years were pooled into one data set because no significant differences in size were observed (t-test, $p > 0.05$). The whole dataset was homoscedastic (Levene's test, $p > 0.05$) and it did not deviate significantly from the normal distribution (Kolmogorov-Smirnov test, $p > 0.05$) (Zar, 1999). The estimation of weight-length relationships was made by adjustments of a power function of the form:

$$W = aBD^b \text{ (Allometry equation).}$$

This equation can be expressed in its linearized form

$$\log W = \log a + b \log BD,$$

where W represents either DW or AFDW for either bell or gonads (it depends on each relation) and BD is the bell diameter. The coefficient 'a' is the intercept and 'b' is the slope (relative growth rates of the variables). The parameters of the weight-length relationships were estimated by linear regression analysis (least squares method).

In order to determine if the values of 'b' obtained in the linear regressions were significantly different from the isometric value ($b = 3$), a t-test (Ho, $b = 3$) was applied, expressed by the equation

$$ts = (b - 3)/Sb,$$

where ts = t-value, b = slope, Sb = standard error of the slope (Sokal & Rohlf, 1987). A t-test was also used to determine significant differences in slopes between two straight lines.

In the field, daily specific growth rates of *O. sambaquiensis* were estimated from mean AFDW with time, which were calculated according to the equation

$$G = ln(W_1/W_0)/t_1 - t_0,$$

where W_1 and W_0 are the mean AFDW (expressed in grams) at time 0 and time 1 respectively (Lucas, 1996; Ishii & Båmstedt, 1998). Daily specific growth rates were calculated separately for bell, gonads, and the whole individual. The mean daily growth was estimated according to the equation

$$C = G_t \times W_t,$$

where G_t is the specific growth rate and W_t is the average AFDW (g) at t (Frandsen & Riigård, 1997).

Morphometric variables, such as BD and GI, were related to the four maturity stages of *O. sambaquiensis* proposed by Macchi et al. (1995): Stage 1 = immature, Stage 2 = early maturation, Stage 3 = mature, Stage 4 = spent. One-way ANOVA and subsequent multiple comparison tests (LSD) were used to determine whether significant differences existed among mean values at every maturity stage. A t-test was used to determine size differences between males and females.

Results

Olindias sambaquiensis medusae appear seasonally in Blanca Bay between November and April

(austral spring, summer and the beginning of autumn). The bell diameter of medusae increased from November to February. Recruitment was high during November, when all individuals had a BD smaller than 4 mm and no gonads (Fig. 3(a) and (b)). However, immature individuals (size class 10–20 mm bell diameter) were observed through January (middle summer). During this period, the water temperature increased from 13.5 to 24 °C (Fig. 3(c)).

The average BD values of *O. sambaquiensis* increased sharply from November (mean BD: 1.33 mm, SD 0.107) to February (summer) (BD: 76.2 mm, SD 2.8) and during this month, the largest medusae (BD: 101 mm) were collected. The mean BD decreased during March (BD: 70.4 mm, SD 2.04) and April (BD: 58.6 mm, SD 4.2) (Fig. 3(a) and (b)). During this period, degradation of the bell, loss of the manubrium, and increasing opacity of the mesoglea was apparent.

From January through February, *O. sambaquiensis* showed the highest relative increase of DW (g) and AFDW (g) within both gonads and bell (Table 1). A maximum relative decrease in BDW (g), BAFDW (g), and GAFDW (g) was observed from February to March (Table 1), while the highest decrease in GDW (g) occurred from March to April. The relative monthly weight increase (and decrease) was always higher in gonads than in the bell (Table 1). The increase of DW and AFDW observed in January and February, coincided with the period of gonad maturation, while the decrease observed during March and April coincides with the spawning.

Proportionally, GDW (as %WW: 13.2%, SD 5.6) and GAFDW (as %DW: 58.2%, SD 8) showed higher values than BDW (as %WW: 4.1%, SD 1.02) and BAFDW (as %DW: 41.5%, SD 7.7). The GDW was positively correlated with BD (significant correlation, $r = 0.374$, $p < 0.01$), while BDW remained constant ($p > 0.05$) as BD increased (Fig. 4(a)). BAFDW decreased as BD increased (significant correlation $r = -0.213$, $p < 0.01$), while no correlation ($p > 0.05$) was apparent between %GAFDW and BD (Fig. 4(b)).

Interestingly, the observed trends of increasing GDW (%WW) and constant GAFDW with size were reflected in those for the whole animal, since TDW (%WW) increased with diameter (significant correlation, $r = 0.32$, $p < 0.01$), whereas TAFDW (%DW) remained constant (no significant correlation $p > 0.05$) as diameter increased.

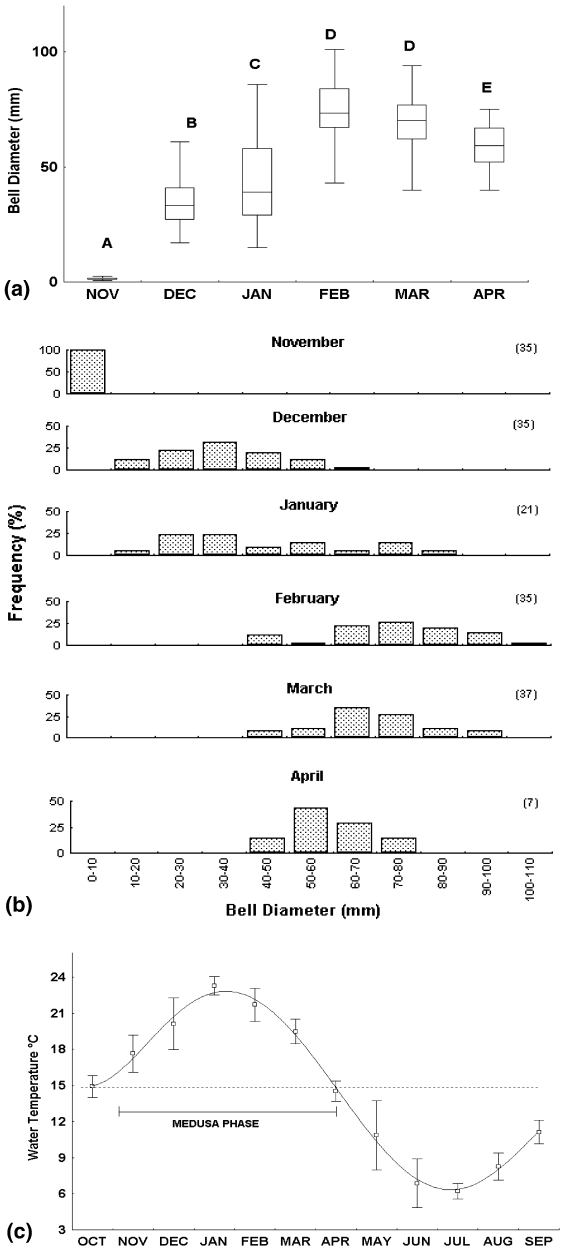

Figure 3. (a, b) Size and frequency distribution of medusae in southern Buenos Aires (Boxes: median, 25–75 percentile, whiskers SE). The numbers on the top right (b) represent the number of individuals sampled during each month from 1982 to 1984. c. Cycle of surface water (1 m depth) temperature in southern Buenos Aires along 1 year. Bars represent standard deviations. The period of occurrence of *Olindias sambaquiensis* medusae in the area of study is shown.

Table 1. Relative monthly changes in DW and AFDW within bell and gonads of *Olindias sambaquiensis*.

PERIOD	BELL		GONADS	
	DW (g)	AFDW (g)	DW (g)	AFDW (g)
DEC–JAN	130.3%	137.8%	297.6%	307.2%
JAN–FEB	**232.7**%	**280.1**%	**544**%	**593.6**%
FEB–MAR	**−24.9**%	**−38.7**%	−27.28%	**−36.22**%
MAR–APR	−19.3%	−34.8%	−22.9%	**−41.4**%

Bold numbers represent either the period of maximum increase (positive numbers) or decrease (negative numbers)

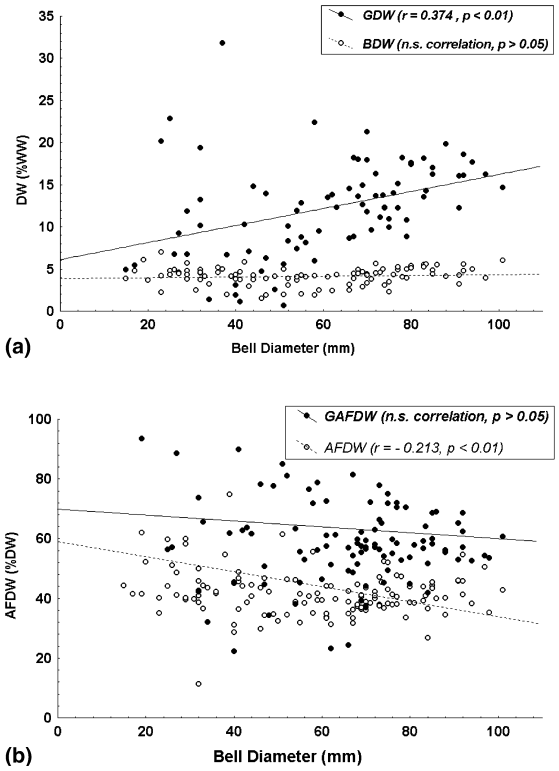

Figure 4. (a) Correlations between bell diameter (BD) and dry weight (DW as % of wet weight). (b) Correlations between bell diameter (BD) and ash-free dry weight (AFDW as % of dry weight). Both correlations calculated for bell (BDW, BADW, solid circles) and gonads (GDW, GAFDW, black circles) of *Olindias sambaquiensis*.

Length-weight relationships

The linear regressions (Fig. 5(a)–(c)) showed that for every length-weight relationship, between 80 and 96% of the variation in weight (g) was a function of the variation of BD. Dependent variables such as GDW, AFDW, TDW, and TAFDW showed a positive allometric relationship with BD (*t*-test, $p < 0.05$) (Fig. 5(a)–(c)). However, BDW and BAFDW were the only dependent variables that maintained an isometric relation with BD (*t*-test, $p > 0.05$) (Fig. 5(a) and (b)). Therefore, it was evident that the observed positive allometry between the total weight-length and bell diameter was due to the contribution of the gonad weight (allometric growth) (Fig. 5(c)).

Moreover, the '*b*' coefficient of the linear equation of the relationships 'GW-BD' (Fig. 5(a) and (b), black lines) was significantly higher than that of the relation 'BW–BD' (Fig. 5(a) and (b), dotted lines) (*t*-test, $p < 0.05$). In other words, the weight of gonads increased at relatively higher rates than that of the rest of the body (Bell).

Specific growth rates and mean daily growth

Before maturation, during November and December, the daily specific growth rates (expressed as growth increment of AFDW) ranged between 13.7 and 28.2% day^{-1} (BD increase 0.03–3.9 mm day^{-1}), while during January and February (beginning of the maturation), the growth rates decreased considerably, ranging between 3.5 and 4.6% day^{-1} (BD increase 0.4–0.8 mm day^{-1}) (Fig. 6). March-April was a period of 'degrowth,' corresponding to values between −1.1% and −1.6% day^{-1} (BD decrease 0.2–0.36 mm day^{-1}) (Fig. 6).

Differences in growth rates of gonads and bell were apparent among specimens during each month, and the daily specific growth (and degrowth) rates for gonads were always higher than those for the rest of the body (Bell) (Fig. 6).

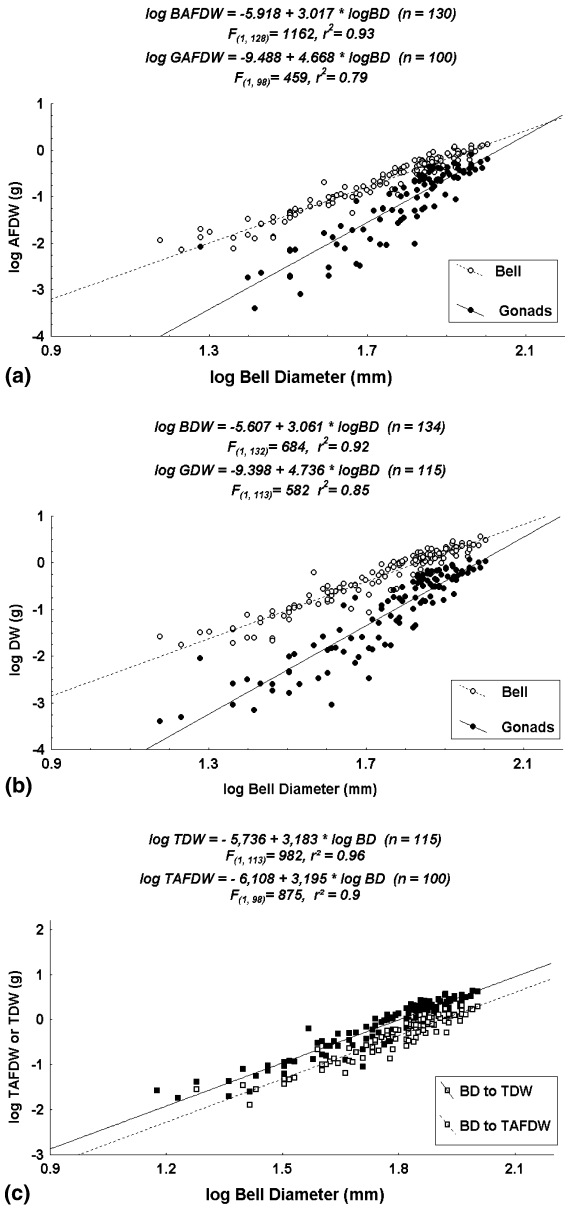
log BAFDW = -5.918 + 3.017 * logBD (n = 130)
F(1, 128)= 1162, r2= 0.93
log GAFDW = -9.488 + 4.668 * logBD (n = 100)
F(1, 98)= 459, r2= 0.79
log AFDW (g)
Bell
Gonads
(a)
log Bell Diameter (mm)
log BDW = -5.607 + 3.061 * logBD (n = 134)
F(1, 132)= 684, r2= 0.92
log GDW = -9.398 + 4.736 * logBD (n = 115)
F(1, 113)= 582 r2= 0.85
log DW (g)
Bell
Gonads
(b)
log Bell Diameter (mm)
log TDW = - 5,736 + 3,183 * log BD (n = 115)
F(1, 113)= 982, r2 = 0.96
log TAFDW = - 6,108 + 3,195 * log BD (n = 100)
F(1, 98)= 875, r2 = 0.9
log TAFDW or TDW (g)
BD to TDW
BD to TAFDW
(c)
log Bell Diameter (mm)

Figure 5. Linear relationships of the bell diameter (BD) to (a) ash-free dry weight (AFDW in grams), (b) dry weight (DW in grams), and (c) total dry weight and total ash-free dry weight (TDW, TAFDW in grams) of *Olindias sambaquiensis*., Gonads (GDW, GAFDW, black circles), bell (BDW, BAFDW, open circles), whole individual (TDW, TAFDW open, and closed squares).

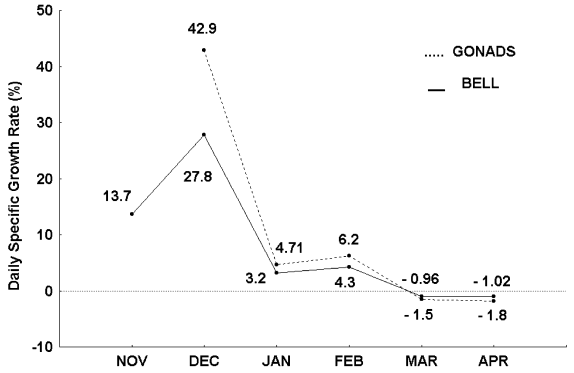

Figure 6. Daily specific growth rate (as increments of AFDW, in grams) for bell (solid line) and gonads (dotted line) of *Olindias sambaquiensis*.

Morphometric indices (BD and GI)

Every maturity stage of the gonad cycle of *Olindias sambaquiensis* was characterized by morphometric variables such as BD (Fig. 7(a)) and GI (Fig. 7(b)). There were no significant differences in size and weight between males and females from the same maturity stage (*t*-test $p > 0.05$).

There were significant differences among BD of the different maturity stages (ANOVA, $F_{(3, 114)}$ 82.5, $p < 0.001$) (Fig. 7(a)). The average values of BD increased from Stage 1 (mean: 32.5 mm, SD 9.3) to Stage 3 (mean: 78 mm, SD 11.4). The smallest mature medusa recorded had a bell diameter of 47 mm. The BD of spent medusae (mean: 70 mm, Stage 4) was significantly smaller (10.25%) than that of mature medusae (Fig. 7(a)).

As expected, the GI increased considerably from the first maturity stage (immature) to the third one (maturation). With the exception of stages 2 and 4, significant differences were observed between maturity stages (ANOVA, $F_{(3, 110)}$ 112.3, $p < 0.001$) (Fig. 7(b)). In immature individuals (maturity stage 1), gonads represented only 4.9% (range 1–7.4%) of TAFDW, while in sexually mature individuals (maturity stage 3), the GI increased considerably to the highest average value of 31.4% of TAFDW, and up to 75% in some individuals. After the release of gametes (maturity Stage 4), the GI decreased to a mean value of 24% (range: 3–60%) (Fig. 7(b)).

A significant positive correlation was observed between GI and BD ($r = 0.658$, $p < 0.01$). A high level of variability was observed between GI and BD (Fig. 7(c)), which is probably due to the small sample size.

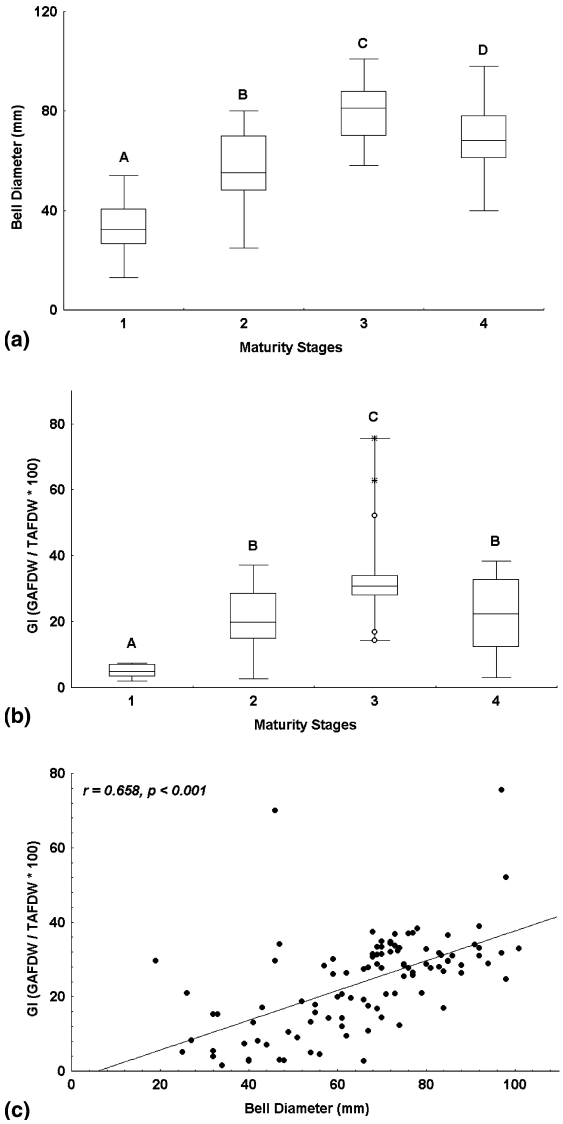

Figure 7. (a) Bell diameters of *Olindias sambaquiensis* at each maturity stage. Letters A–D indicate significant differences (ANOVA, LSD test) at $p < 0.05$ level. (b) Gonad index (GI) of *Olindias sambaquiensis* in relation to maturity stages. Boxes: median, 25–75 percentile, whiskers max–min. Circles, outliers; asterisks, extreme values. Letters A–D indicate significant differences (ANOVA, LSD test) at $p < 0.05$ level. (c) Correlation between the GI and the bell diameter.

Discussion

This is the first study to evaluate the relationship between growth of gonad and somatic tissue during the development on the medusa stage in a hydrozoan life cycle. Previous studies have been carried out in the scyphozoans *Aurelia aurita* and *Chrysaora fuscescens* (Möller, 1980; Shenker, 1985; Lucas, 1996; Ishii & Båmstedt, 1998) that have internal gonads surrounded by gastric tissues, which are not easily separable from the rest of its body. Similarly, studies on growth of hydromedusae have dealt with species that are small (i.e. *Clytia* sp., *C. hemisphaerica* and *Cladonema californicum*) (Costello, 1991; Matsakis, 1993; Lucas et al., 1995, respectively), from which gonads cannot be readily separated from the bell. In contrast, *Olindias sambaquiensis* is a large hydromedusa, which can reach up to 210 mm of bell diameter (Mianzan & Ramírez, 1996), and its gonads are conspicuous structures that hang from the radial canals (Kramp, 1961) and are therefore easily separated from the rest of its body. These features make it an ideal experimental model.

Although the growth pattern of *O. sambaquiensis* in Blanca Bay area shared the three general characteristics previously described for other species (see Arai, 1992; Lucas, 1996; Ishii & Båmstedt, 1998), the present study shows, for the first time, a positive allometric growth pattern in medusae that is caused by the allometric growth of gonads. The slope values of the length-weight (TDW and TAFDW) relationships obtained for *O. sambaquiensis* (Fig. 5(c)) are higher than those previously reported for other hydromedusae and scyphomedusae species (see Lucas et al., 1995; p. 365, Fig. 4; Hirst & Lucas, 1998; p. 261, Table 1; Jankowski, 2000; p. 1335, Table 3).

Although both food availability and temperature would play a role in regulating and/or determining an allometric gonad growth in *O. sambaquiensis* (Matsakis, 1993; Ishii & Båmstedt, 1998), other factors, such as interactions between the environment and specific genes, could be also very important. Unfortunately, this subject remains unexplored in cnidarians.

The allometric increase of TDW and TAFDW in the weight of *O. sambaquiensis* medusae is a consequence of a high energy investment into reproduction (gonad weight) rather than somatic growth. GI (AFDW) values in mature individuals was 31%, reaching in some extreme cases up to 75 %. High GI values, similar to those obtained in the present study, have been observed only in other hydromedusae species (Larson, 1986b), while in the scyphomedusa *Aurelia aurita* the GI values are

relatively low (Lucas & Lawes, 1998). Moreover, the loss of weight in gonads in spent medusae of *O. sambaquiensis* represented the amount of 'total organics' (GAFDW) invested (up to 41.4%) in gamete production. Thus, *O. sambaquiensis* can lose a substantial portion of its TAFDW (g) (up to 28%, data not shown) due to the release of gametes only. These results corroborate the results obtained by Macchi et al. (1995): the thickness of the germinal epithelium of both males and females of *O. sambaquiensis* decreases by about a half after the release of gametes (stage 4, spent individuals).

Until now, Lucas (1994) carried out the only study on biochemical composition of gonads and umbrella of a medusa species (on the Scyphozoan *Aurelia aurita*) related to size and sexual maturity. In this species, the BAFDW (%DW) values remain near constant, whereas GAFDW decreased with medusa size, thereby affecting the 'total organics' (TAFDW) – size relationship. Our study shows a different relationship between the weight of gonads and the rest of the medusa; a negative correlation between BAFDW (%DW) and size, and steady GAFDW (%DW) values throughout medusa growth, and due to this last fact, the TAFDW (%DW) also remains constant. These differences may be caused by a discrepancy between the 'total organics' used by Lucas (1994) and TAFDW used in the present study. 'Total organics' represents just a portion (sometimes small) of the TAFDW. Therefore, they might not be comparable.

Our results show the same patterns as those found by Arai (1986) for the hydrozoan *Aequorea victoria*, where the concentration of 'total organics' (TAFDW) remains constant as the medusa increases its bell diameter. In addition, Shenker (1985) observed that mature individuals of *Chrysaora fuescescens* presented a higher TDW (%WW) than immature animals. Our data also support his findings, since TDW (%WW) in *O. sambaquiensis* increases with diameter (mature individuals are significantly larger than immature ones: Fig. 7(a)). The limited available information suggests that relationships between somatic growth and gonad development may be different between scyphomedusae and hydromedusae. Further studies are needed to clarify the relationships between somatic and gonad growth patterns in scyphozoans and hydrozoans.

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Reproduction of the colonial hydroid *Obelia geniculata* (L., 1758) (Cnidaria, Hydrozoa) in the White Sea

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Key words: *Obelia geniculata*, White Sea, life cycle, asexual propagation, frustules

Abstract

Populations of the colonial hydroid *Obelia geniculata* in the White Sea reproduce asexually by frustule formation. Young medusae appear in the plankton during July and August. The number of medusae rarely exceeds 36 per m^3, and the average number varies every year from 0.4 to 10 per m^3. The size of medusae is smaller than reported from other regions. The umbrella of the largest recorded medusa was only 0.57 mm in diameter and the specimen had just 35 tentacles. Only a few mature medusae were found during the study. The colonies in the White Sea are epiphytic and grow only on laminarian thalli. At the beginning of July there are no colonies on thalli from the upper subtidal zone. By the end of August, colonies of *O. geniculata* had increased in density to 30 per m^2. Hydroid recruitment was attributed to active frustule production by colonies living below that zone. The frustules detach from the stems of the hydroids and are found in plankton. Production of frustules on branches occurs continuously during colony growth until water temperatures climb above 0 °C. We found that water temperature in this Arctic environment is generally too low for medusa maturation and planula development in the species. Propagation by frustule formation is the principal means of reproduction in *Obelia geniculata* within the White Sea, and this phenomenon accounts for the species being a dominant epiphyte on laminarian thalli there.

Introduction

The life cycle of *Obelia geniculata* (L., 1758) includes free-living medusa, planula, and sessile colonial polyp stages (Fig. 1). This species is reported to have a worldwide distribution. Colonies have been reported in shallow waters from areas such as Australia (Watson, 1992), South-Eastern Brazil (Grohmann et al., 1997), North America, the North Sea, and the Mediterranean Sea (Naumov, 1969) on various substrates, commonly on stones. The northeast limit of the species natural range in the Arctic region is the White Sea and the Barents Sea. Details of the biology of this species at the northern limits of its natural distributional range have not been investigated to any extent.

Asexual propagation is known in many species of hydroids (Ivanova-Kazas, 1977). One such method in species referable to the genus Obelia is frustule formation. Nevertheless, the biological and ecological implications of this process are still little known. As a rule authors have drawn attention to morphological aspects of this phenomenon (Berrill, 1948, 1949; Auberson & Tardent, 1980; Carrè & Carrè, 1990).

Panteleeva (1999) found that development of medusa gonads in *O. geniculata* requires a water temperature of not less than 15 °C. Conditions in the White Sea are far from this minimum. The aim

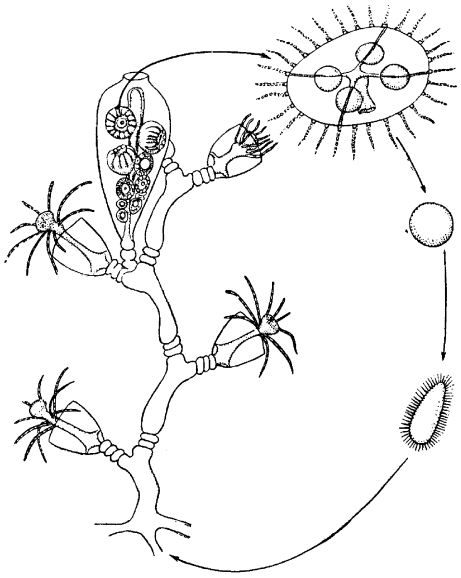

Figure 1. Typical life cycle of *Obelia geniculata* (after Naumov, 1969).

of our study was to investigate how reproduction of *O. geniculata* occurs under such unfavorable environmental conditions. In doing so we noted the peculiar distribution of the species in the White Sea, where it occurs only on laminarian thalli. We examined this phenomenon by studying the whole life cycle of the species and by observing its settlement on substrates in the study area. Our results indicate that a peculiar mode of reproduction in response to sub-optimally cold conditions accounts for these observations.

Materials and methods

Study material was collected from Kandalaksha Bay, northern Russia, near the White Sea Biological Station (66° 33′ 18″ N, 33° 06′ 22″ E). Plankton samples, at depths of 0–10 m, were made using a plankton net. Quantitative living plankton samples were checked immediately for medusae and frustules in laboratory. Colonies on laminarian thalli were collected manually from the subtidal zone (0.5–7.0 m) by scuba divers or with a drag sampler. The investigation was carried out during the summer seasons of 1999–2002.

Results

Medusae

Medusae were recorded in the plankton during the study as early as 27 June 2002, and were present until the end of September. In 2000 and 2001 they were not recorded in the plankton until early August. Their densities in plankton vary during the summer for the most part from 0 to 8 per m^3, and rarely exceed 10 per m^3 (Table 1). Most of them have no gonads on the radial canals and are small in size. The average diameter of the umbrella is 0.35 mm and there are about 25 tentacles (Fig. 2(a)). The largest specimen was about 0.5 mm and there were 32 tentacles on the margin of the umbrella. We did not find any mature medusae like those described by Mayer (1910) and Naumov (1969) (Fig. 2(b)), which can reach 6 mm in diameter and have more than 100 tentacles.

Colonies

Colonies of *O. geniculata* were found only on laminarian thalli in shallow subtidal waters (0.5–7 m depth), though there were other common substrates available. At the beginning of summer (June) there were no colonies on thalli from the upper subtidal region, and not more than 1–3 per m^2 on those from deeper waters. At the moment, they arranged only on the distal part of thalli. By

Table 1. Numbers of medusae of *Obelia geniculata* in plankton from the study area in the White Sea (per m^3)

Year	Date																	
	12.7	15.7	18.7	21.7	24.7	27.7	30.7	2.8	5.8	8.8	11.8	14.8	17.8	20.8	23.8	26.8	29.8	1.9
2002	0	1	10	5	12	0	3	8	23	10	3	1	3	3	5	4	4	3
2001										0	2	1	2	0	1	2	1	0
2000									4	6	–	8	2	0	20	4	36	

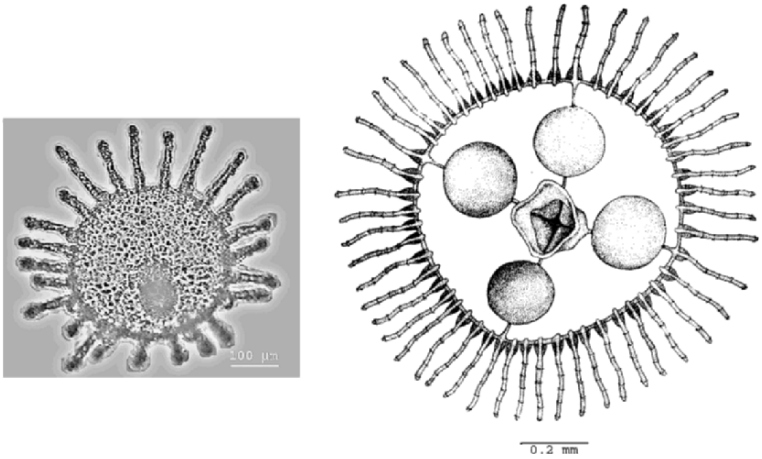

Figure 2. Medusae of *Obelia geniculata*. Left: From the White Sea. Right: After Naumov, 1969.

the end of August, however, colony density of *O. geniculata* had increased to as much as 30 per m^2, and the colonies were distributed evenly upon the laminarian thalli.

Frustules

Frustule formation in the White Sea occurs continuously during growth of the colonies and is very intensive during the summer. The ratio between developing frustules and hydranths on a branch varies between 1:8 and 1:2. Frustules were found in plankton throughout the summer season (Table 2). Their abundances varied from 0 to 25 per m^3. Frustules have a fixed size of about 0.6 mm in length, with variations of less than 50%.

Table 2. Numbers of frustules in plankton from Kandalaksha Bay, White Sea, during 2002 (per m^3)

July (day)	Numbers per m^3	August (day)	Numbers per m^3
3	1	2	9
6	6	5	25
9	6	8	10
12	3	11	3
15	1	14	1
18	10	17	3
21	6	20	3
24	13	23	5
27	0	26	4
30	3	29	4

Ninety-five percent of frustules taken from living plankton samples gave rise to new colonies. Frustules produced colonies only when they made contact with a substrate. Experiments involved cultivation of frustules suspended under the water surface. The anterior end of a frustule is a growth apex, which elongates continuously until it touches some surface and sticks to it.

In our experiments in standing water, frustules attached to any hydrophilic or hydrophobic, or biotic, or abiotic surface including glass, parafilm, plastic, stones, and even the water surface film and fucoid thalli. Attachment time depended on the type of substrate. In our tests, attachment to laminarian thalli took 3–15 (average 5) min, to glass took 7–20 (average 10) min, and to fucoid thalli took from 15 min up to several hours.

Once a frustule has attached, it moves forward on the substrate like a stolon, leaving empty perisarc behind it after 1–3 h. The newly formed growth apex of the stolon is then inhibited by the development of the primary hydranth for another 9–12 h (Fig. 3).

Discussion

We did not find any mature medusae of *O. geniculata* in plankton. The minimum water temperature for development of gonads to occur in medusae of *Obelia geniculata* must be more than 15 °C (Panteleeva, 1999), while the water temperature in the White Sea rarely exceeds this value

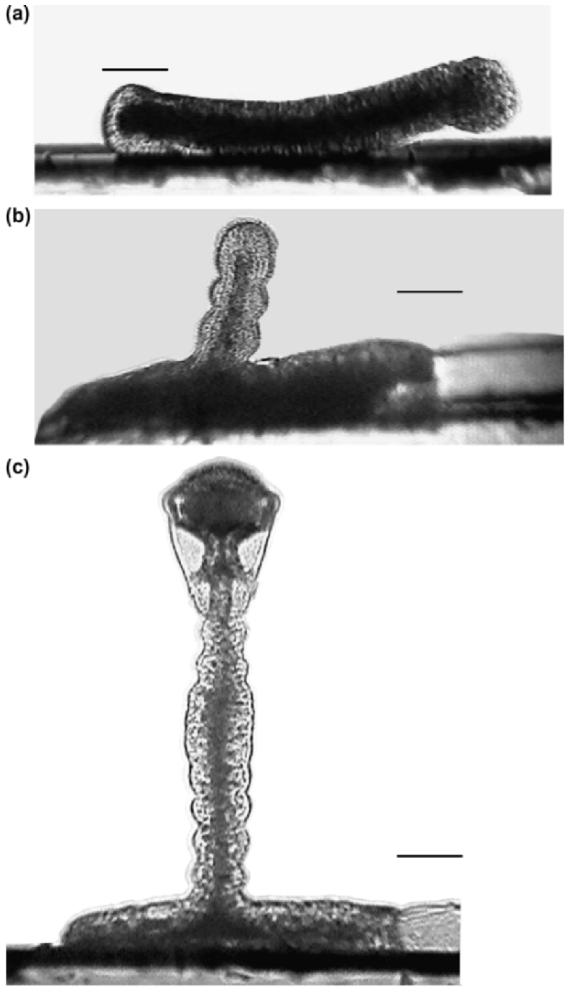

Figure 3. Stages in the formation of a new colony from a frustule (scale, 0.1 mm). (a) Frustule newly attached to glass (side view). (b) The same frustule after 6 h (side view). (c) Newly forming polyp, from frustule 12 h after attachment (side view).

(Fig. 4). Thus seawater temperatures are, for the most part, too low for medusae of *Obelia geniculata* to attain maturity. We believe maturation and sexual reproduction occurs only during exceptionally warm periods. This phenomenon has remained unnoticed because a closely-related species (*Obelia longissima*) also occurs in the White Sea. Medusae of *O. longissima* are almost indistinguishable morphologically from those of *O. geniculata*, and by contrast they undergo a complete life cycle in the study area. Maturation of *O. longissima* medusae occurs at lower seawater temperatures, about 10–12 °C (Stepanjants et al., 1993; Panteleeva, 1999). Their planulae swim and settle on various substrates, even vertical walls and bottoms of ships. This mode of reproduction is unavailable to *O. geniculata* in the White Sea, and an alternative method, frustule formation as described by Berrill (1948, 1949), is essential for survival of the species there. The mode of reproduction like this was described to *Obelia longissima* in the Barents Sea (Panteleeva, 1999) (Fig. 5).

At the beginning of summer (June) a very small number of colonies is found and only on the distal parts and the very small number of colonies of laminarian thalli. Colonies are inactive during winter when water temperatures are below 0 °C (Marfenin & Karlsen, 1983; Calder, 1990). At the same time their substrates (laminarian thalli) continue to grow proximally and deteriorate distally, so that colonies are eliminated as if they were on a conveyor. Every summer colony density increases even when there are no mature medusae in plankton. This increase is attributed to active frustule production by colonies. Frustule production is a normal biological process that is essential to the survival of *Obelia geniculata* in the White Sea. It extends over a longer time period than medusa production, and is more effective as a mode of reproduction in the life cycle. Whereas medusa formation is a reproductive 'dead end' for *O. geniculata* in the White Sea, an estimated 95% of frustules give rise to new colonies. We have found that frustules are a constant component of summer plankton in the White Sea. The presence of frustules in the plankton was unexpected because nobody had previously made note of them. Frustule numbers in the plankton were large enough that their role in the reproduction of species of Obelia is undoubted. This observation was supported by production of frustules on the sessile hydroid colonies. Our plankton samples were taken far from these colonies, confirming our hypothesis of significant spreading by currents. Probably frustulation is cloning in the broad sense of the word. Thus, the local population retains more successful gene modifications. This phenomenon should be considered in analyses of hydroid life cycles.

Experiments on frustules reveal that in standing water they can settle on many substrates. Yet, we found colonies of *Obelia geniculata* only on laminarian thalli. Our previous investigations re-

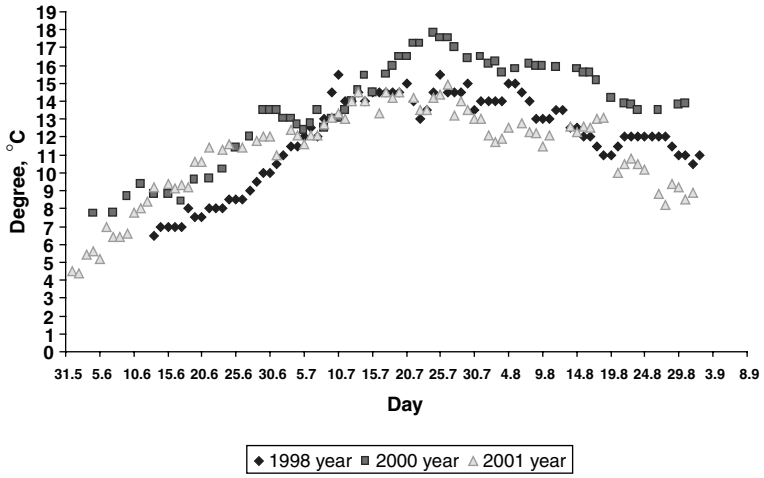

Figure 4. Water temperatures from the G. Salma channel, near the WSBS pier (low tide, at a depth of 1 m).

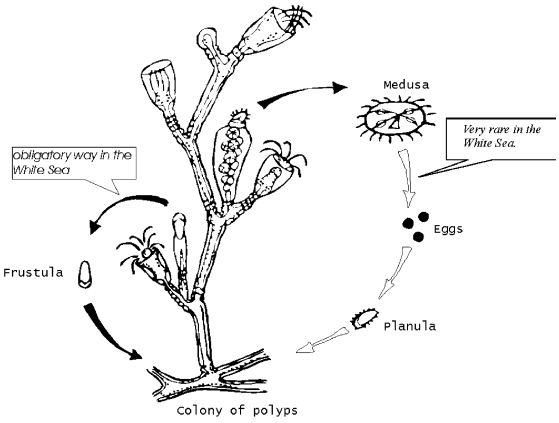

Figure 5. Life cycle of *Obelia geniculata* in the White Sea.

vealed adaptations in colonies of *Obelia geniculata* to existence in strong currents, where it typically occurs (Marfenin, 1984; Maljutin & Marfenin, 1988). We therefore assumed that some selectivity occurs in its substrate by the frustule. Unlike planulae, frustules are covered by perisarc and do not swim. An absence of active settlement adaptations limits the distribution of colonies to laminarian thalli. Frustules settle and attach to their substrate by means of sticky perisarc, and grow slowly. This process requires time even in standing water. Experiments revealed the fastest frustules attachment to laminarian thalli. There is insufficient time, not more than 1 h during slack tides, for planulae to settle under these conditions. Thus, germination of frustules is considered possible only on the surface of laminarian thalli. Frustules remain on the wide and flat mucous-covered blades of the alga for a long time, and even if washed off they adhere to adjacent blades.

Results from investigations on the life cycle of *Obelia geniculata* account for the peculiar distribution of this species in the White Sea, where it occurs predominantly on laminarian thalli. Our results indicate that reproduction by frustules and by medusae actually constitute different ecological strategies. Frustule production leads to cloning of optimal morphotypes and rapid increases in hydroid abundance, which are advantageous for survival under high mortality. Medusae production and subsequent sexual reproduction is of prime importance in effecting genetic diversity. Secondarily, it leads to colonization of new substrates, which presents difficulties for frustules.

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Heterogeneous and compensatory growth in *Melithaea flabellifera* (Octocorallia: Melithaeidae) in Japan

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Key words: sea fan, gorgonian

Abstract

Melithaea flabellifera (Kükenthal, 1909) (Octocorallia, Gorgonacea), an endemic and predominant gorgonian in Japanese shallow waters, grows mostly in one plane and ramifies in a dichotomous way. The growth rates of all branches and of the colony were measured from photographs of tagged gorgonian corals for approximately 1 year from June 1995 to May 1996 on the western Pacific coast of Japan (34° 39′ N, 138° 56′ E). The most rapid growth occurred when the water temperature was less than 20 °C. Mean growth ranged from 2.9 to 11.4 mm year^{-1}. Linear growth of individual branches ranged between −30.4 and 24.8 mm year^{-1}. Both seasonal and non-seasonal variations in growth rate were observed in each colony. When branches were lost, the adjacent branches grew faster, filling the open space in the fan. Heterogeneity in growth rate within a colony was partly caused by this compensatory growth. This indicates that the regular branching pattern of gorgonians is due to irregular and heterogeneous growth. The compensatory growth suggests that *M. flabellifera* is constrained by some potential optimal size or form.

Introduction

The growth forms of arborescent marine invertebrate organisms (corals, hydroids, bryozoans, and sponges) have been studied in detail. Growth in colonial modular organisms has been termed 'indeterminate' (Jackson, 1977; Jackson et al., 1985, 2001; Sebens, 1987; McKinney & Jackson, 1989; Coma et al., 1998b), suggesting that organisms could theoretically avoid the constraints of size or longevity that single-module organisms cannot. Actual examples of unlimited growth have been reported (Buddemeier & Kinzie, 1976; Hughes & Jackson, 1985; Hughes et al., 1992). On the other hand, it has been found that there are environmental, genetic (Sebens, 1987), and mechanical (Sebens, 1982, 1987) constraints limiting indeterminate growth.

The octocoral order Gorgonacea is one of the most conspicuous groups that forms colonies by the iteration of polyps as modules and exhibits regular growth forms. Suspension feeders, gorgonians are common in areas characterized by strong, fast-flowing currents and sometimes by upwelling (Reed, 1983; Sebens, 1984). Gorgonians may play a significant role in energy transfer between planktonic and benthic communities (Mistri & Ceccherelli, 1994; Heikoop et al., 2002; Risk et al., 2002; Orejas et al., 2003). The forms of gorgonians depend strongly on this feeding strategy. Although the fan shape of many species appears to have symmetrical branching, the iterative growth of these branches is not continuous and homogeneous. It has been reported that each branch grows independently (Velimirov, 1975; Weinberg & Weinberg, 1979;

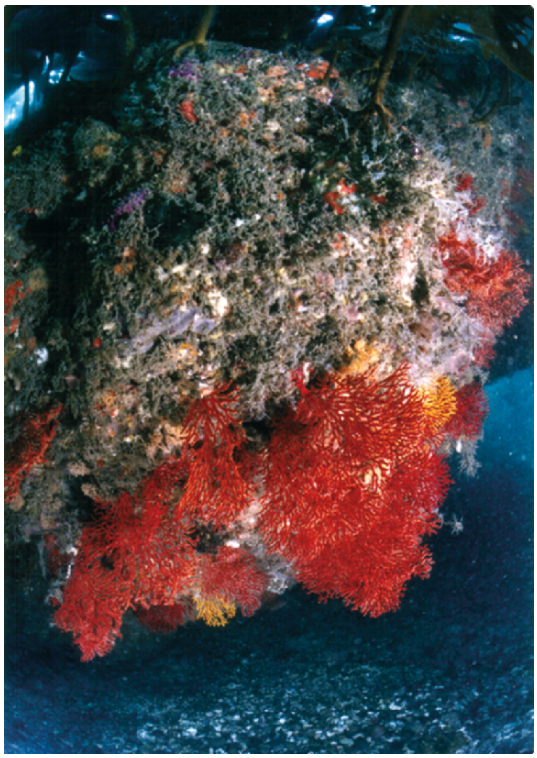

Figure 1. Melithaea flabellifera (Kükenthal, 1909) colonies on the lower surface of an overhanging ledge (1–5 m deep) at Shimoda, Izu Peninsula, Japan (Photo Ochi).

Brazeau & Lasker, 1992; Castanaro & Lasker, 2003).

Gorgonians are common in rocky benthic communities along the Japanese coast in habitats ranging from coral reefs of the subtropical regions to subarctic zones, and from shallow water to depths of over several thousand metres. Although the group exhibits a rich diversity in Japan, only a few papers have dealt with the ecology of Japanese gorgonians.

Melithaea flabellifera (Kükenthal, 1909) is an endemic and predominantly shallow-water gorgonian that lives on vertical and overhanging rock surfaces. This species is distributed on both the Pacific and the Sea of Japan coasts in temperate Japan. The colonies, which exhibit intense hues of red (or orange, yellow, or pink), grow mostly in one plane and ramify dichotomously (Kükenthal, 1909; Fig. 1).

This paper describes the seasonal and non-seasonal heterogeneous growth rates within a colony and population of *M. flabellifera*. It also discusses the regular morphological form of branching gorgonian corals that results from heterogeneous growth, and the relationship between growth rate and form.

Materials and methods

Fieldwork was conducted at Shimoda, on the Izu Peninsula, on the Pacific Ocean coast of Japan (34° 39′ N, 138° 56′ E; Fig. 2). Twenty-one colonies of *Melithaea flabellifera* were randomly selected in the same locality and each was tagged with a numbered, plastic tape tags wired to its base. Seven colonies were tagged in June 1995, and 13 in August 1995. One colony detached and therefore another was tagged in November 1995. Photographs were taken once or twice of the whole colony, and close-up colony images for branch measurement were taken 25 times at weekly-to-monthly intervals during the period June 1995–May 1996. The colonies marked in June 1995 were not suitable for estimation of growth rate because of branch overlapping, and therefore were used only for measurement of colony height, area, and number of branches. Six of the colonies marked in August 1995 were used for growth rate measurement (Table 1).

Linear growth was determined by measuring the length of the branch tips in close-up images and calculating the length increments. Because a fan is often slightly curved, each marked colony was gently compressed between a clear acrylic plate and a white plastic sheet marked with a 1-mm grid. It was photographed *in situ* at right angles to the plane. Pentagonal plates were used to fit under overhangs and against diagonal walls where the gorgonians lived. Computer-imaging analysis tools were used to capture, compose, and calibrate images of each colony and to measure the colony and branches. To estimate entire colony size, height and area were measured as described by Weinbauer & Velimirov (1995, 1998). In this study, height is defined as the distance between the base and the farthest tips. A line was drawn between neighbouring branch tips at the periphery of each colony and the area within this outline was determined. To estimate growth rate, all branch tips of the entire colony were measured along the medial axis from the point opposite its origin

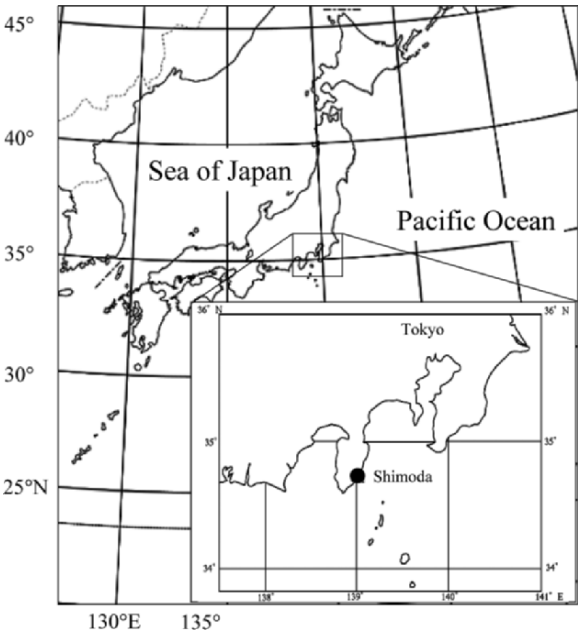

Figure 2. Study location, Shimoda, Izu Peninsula, in the temperate zone in the western Pacific Ocean on the east coast of Japan (34° 39′ N, 138° 56′ E).

(Brazeau & Lasker, 1988, 1992), and those measurements were regarded as the linear growth of skeletal axes. The number of tips of a branch was used for the index of fan formation. Growth rate was calculated based on the growth of the tips of a branch divided by the number of days of observation. The calculated data were plotted to the middle date (Figs 3, 4(B) and 5(B)) between two dates when the branch length increments were measured. Therefore, the data recorded in August 1995 and May 1996 were plotted in the figures for late August and April. All measurements were to an accuracy of 0.1 mm.

From the same community where the growth measurements were made, some colonies were sampled to study reproduction. Samples were collected between March and May 1996 and observed in the laboratory. Considering the probability that planulae-shedding was stimulated by collection, *in situ* observations were also conducted using photographs on the same day that planula release was observed in the laboratory. Direct or video observations were difficult because of suspended matter: planulae could not be distinguished from debris in the water. Data on water temperature were obtained from the 'Quick Bulletin of Ocean Conditions' published by the Hydrographic and Oceanographic Department of the Japan Hydrographic Association, Japan Coast Guard, in 1995 and 1996.

Results

The top of the rocky shallow shore is visually dominated by brown algae, and under the seaweed a dense population of gorgonians thrives on the shoals (Fig. 1), on overhanging or vertical walls with their tips pointing downward at depths of about 1–5 m.

Table 1 shows colony height, area, number of branches, mean growth rate, and range of linear growth length. This result shows that mean growth rate underestimates the growth variation within a colony that actually occurred. The linear growth of each branch ranged very widely. The maximum

Table 1. Growth of *Melithaea flabellifera* measured between June 1995 and May 1996

Colony no.	Height (mm)	Area (mm^2)	Number of branch tips	Mean growth (mm/year/branch)	Growth range (mm)
2	124.8	7499	65	11.4	−30.4; 24.8
4	127.2	11087	77	3.7	−5.6; 14.8
7	121.2	12566	93	2.9	−6.8; 10.4
12	51.1	1335	38	10.2	−2.9; 22.7
18	103.9	7059	84	3.7	−12.8; 18.6
20	64.3	2171	45	6.5	−2.8; 22.4

Mean growth per colony per year was measured as the average growth of individual branches. The date to which the height, area, and number of branches figures pertain is 8 September 1995 for colonies 2, 4, and 18, 1 July 1995 for colony 7, and 15 August 1995 for colonies 12 and 20.

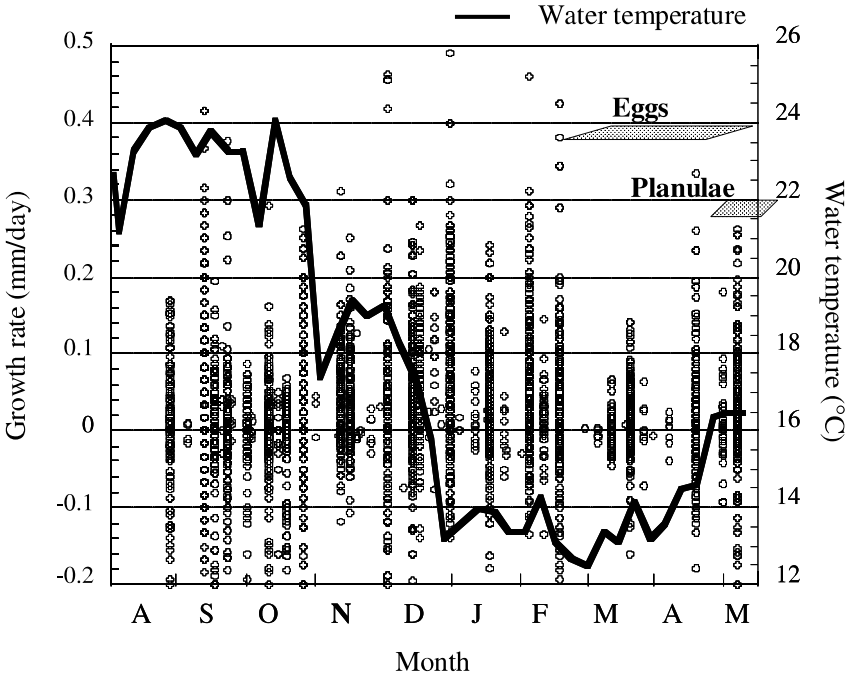

Figure 3. Variation in growth rate within a colony and within a population. Growth rates of all tips of branches in the six colonies in Table 1 were plotted from August 1995 to May 1996. Eggs or planulae were observed in the period shown by the stippled trapezoids.

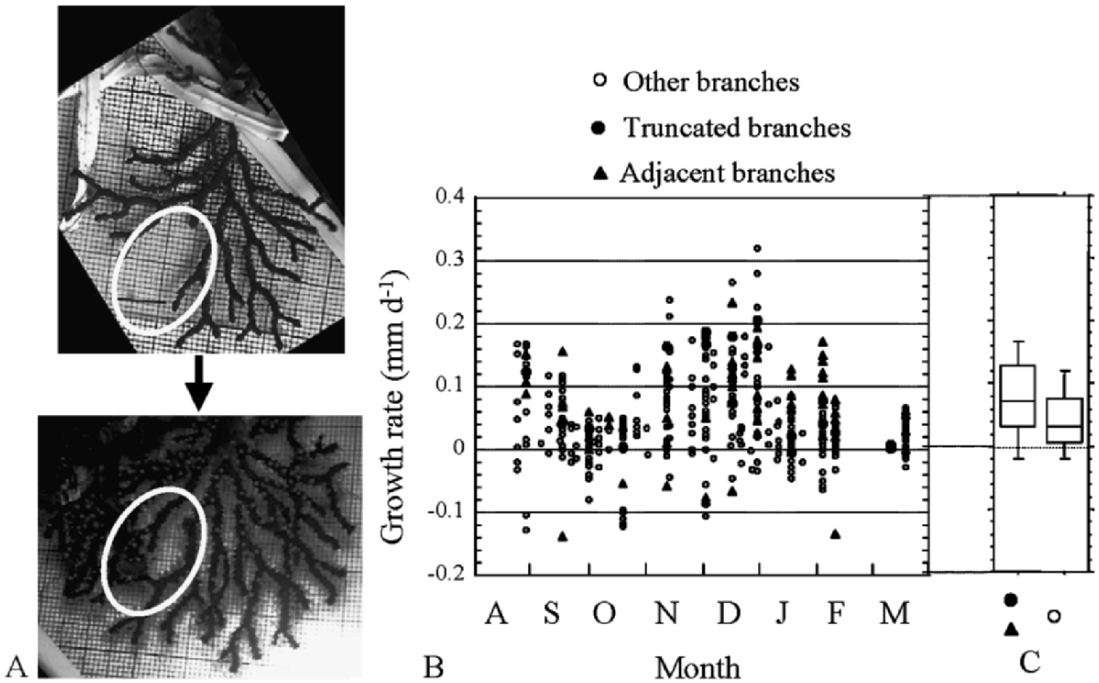

Figure 4. Typical observation of compensatory growth of adjacent branches in *Melithaea flabellifera* colony 12 (all measurements included). (A) Lost branch (upper) and grown branches (lower). (B) Growth rate July 1995–May 1996. (C) Box plot of growth rate variation by branch type. Box is the interquartile range with median line and 10% and 90% bars.

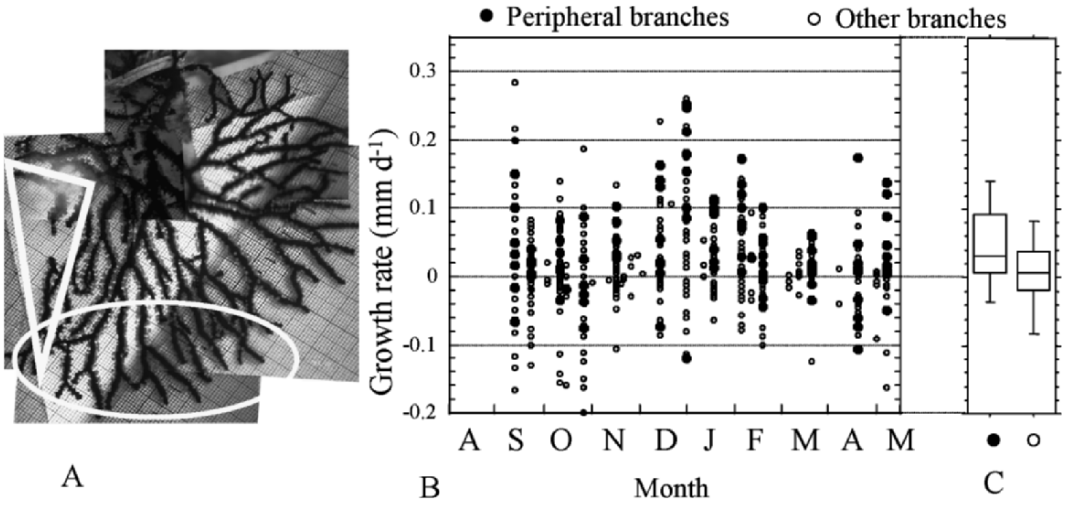

Figure 5. Typical observation of compensatory growth to fill empty space in the fan in *Melithaea flabellifera* colony 4 (all measurements included). (A) Branch area lost in November 1995 (triangle) and peripheral branches (oval). (B) Growth rate July 1995–May 1996. (C) Box plot of growth rate variation by branch type. Box is the interquartile range with median line and 10% and 90% bars.

number of branches per colony was 204. Mean growth rate ranged from 2.9 to 11.4 mm year^{-1}; growth range of individual branches was between −30.4 and 24.8 mm year^{-1} (Table 1).

Figure 3 shows growth rates of all branch tips of the six colonies listed in Table 1. Symbols represent the growth rate of branches in different colonies. The maximum growth rate was observed from November 1995 to the end of February 1996. The water temperature was below 20 °C from November 1995 to June 1996. Rapid growth was observed in winter when the water temperature was less than 20 °C. However, it was not observed in early summer when the water temperature was also less than 20 °C. The high growth rate period may have continued until the middle of March 1996, because no images were recorded between late February and early April 1996.

The decreased branch length shown in Figure 3 is mainly accidental negative growth caused by the dropping off of branches. The largest detachment was not due to breakage of the colony base, but rather to the loss of an entire colony caused by collapse of the substratum to which it was attached. Partial breakage of branches was observed, but they did not detach from the main axis for about 1 mo. This did not result in the immediate death of the branch. However, the branch did not adhere to the main colony again. The smallest branch losses were several millimetres from the tips. The reason for the small negative growth is not clear. Polyp length is about 1 mm; therefore these decreases were larger than measurement error.

Predation on *M. flabellifera* was not observed in this study, although it was observed at other sites that the surface of *Melithaea* sp. was subject to grazing by gastropods. The plastic markers used to tag the colonies were covered with bryozoans, algae, and other organisms, but these organisms, except for temporary adhesion by algae, did not cover the colonies of the gorgonians themselves.

The colonies measured in this study belonged to a single population. Growth rates were variable among branches within the same colony and varied between colonies (Fig. 3). Typical heterogeneity of growth within a colony is illustrated in Figure 4. Colony 12, depicted in Figure 4(A), was not very large, at only 51.1 mm in height (Table 1), and there was a gap in the middle of the fan (Figure 4(A), upper image). The truncated branch and adjacent branches grew faster to fill the void and generated marked heterogeneity in the growth rates within this colony (Fig. 4(B) and (C)). These branches had grown as much as 22.7 mm by the end of the study. Colony four lost many branches on its left side in November 1995 (Fig. 5(A), triangle). Subsequently, the branches on the periphery (Fig. 5(A), oval) grew faster than the others to increase the fan area again (Fig. 5(B) and (C)).

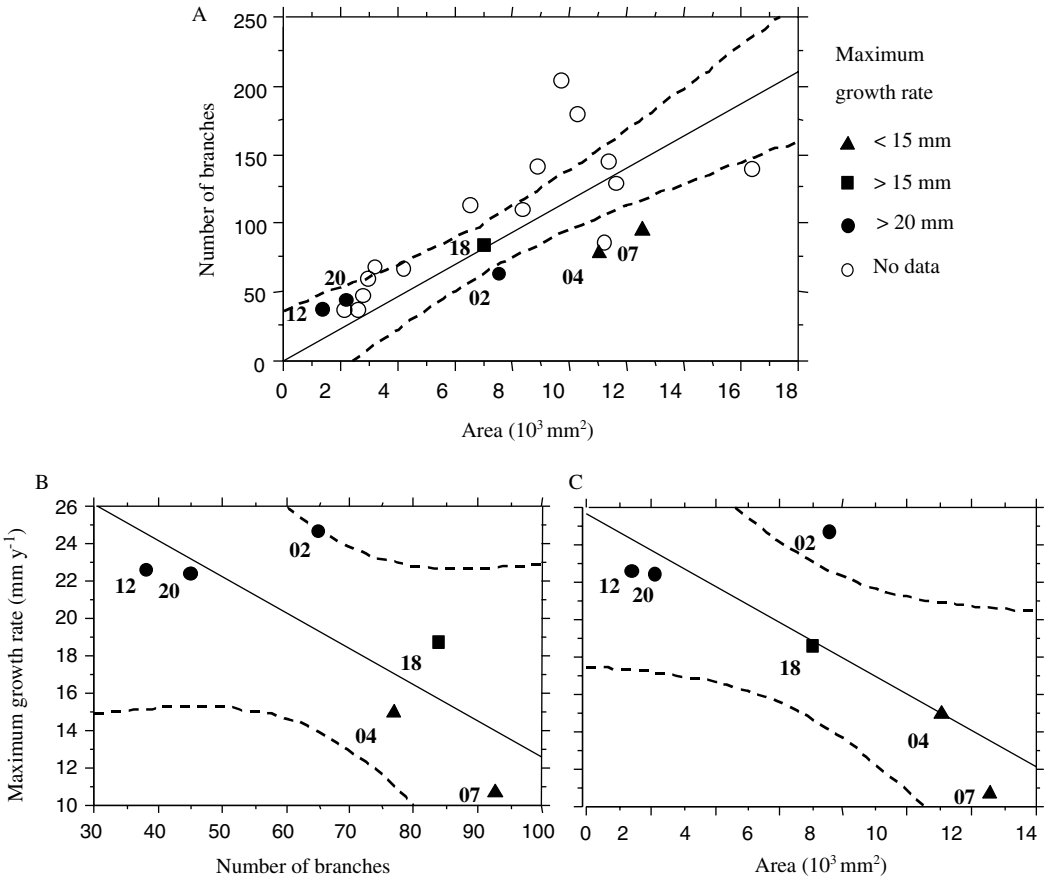

Figure 6. Maximal growth rate, number of branches, and area. (A) Density of branches. Plots and the results from least-squares linear regressions ($r^2 = 0.873$, $p < 0.0001$) (dotted lines are 95% predicted intervals). (B) Number of branches and maximum growth rate. (C) Area and maximum growth rate. The results from least-squares linear regressions for (B) are $r^2 = 0.602$, $p = 0.007$, and for C are $r^2 = 0.637$, $p = 0.001$ (dotted lines are 95% predicted intervals). Numbers refer to the colonies in Table 1.

This colony was 127 mm in height and thus not small for this species (Table 1). The maximum growth of its branches was 14.8 mm.

Data on fan area, number of branches, and maximal growth rate from Table 1 and data on fan area and number of branches from the colonies in which growth rate was not measured are shown in Figure 6. The tendency toward many branches and large area could be explained by colony area ($r^2 = 0.873$, $p < 0.0001$), which supports strong linear relations between area and number of branches (Fig. 6(A)). The number of branches and maximal growth rate ($r^2 = 0.602$, $p = 0.0044$; Fig. 6(B)), and area and maximal growth rate ($r^2 = 0.637$, $p = 0.001$; Fig. 6(C)) also support this tendency, and also support the tendency of larger colonies to grow more slowly (Fig. 6(B) and (C)). Maximum growth rate was used in this study because it indicates growth capability, while the mean includes negative growth.

Gonads were first observed in January 1996, and unfertilised eggs rose to the oral part of the gastrovascular cavity in each polyp in April. The colonies collected in April released unfertilised eggs in the laboratory but all died without developing. Planulae were observed with both unfertilised eggs and blastomeres in a colony in early May (stippled trapezoids labelled 'Eggs' and 'Planulae' in Fig. 3). Each planula was 150–800 μm long and 120–520 μm wide. The range in length was due to different developmental stages and morphologic changes of the planulae, which already had swimming motility. There were 10–29 eggs plus planulae per polyp when the polyps were

dissected (Matsumoto, 1997). At the end of May, planula release was confirmed both in the laboratory and *in situ*. Planulae began to be released 2 days after the water temperature exceeded 18 °C *in situ*. Planulae were released in 1996 at the study site in late May and June. It was not synchronous, differed from colony to colony, and occurred every day. However, the reproductive season was 1 mo later at Tateyama Bay, Japan (139° 49′ E, 34° 59′ N) in the same year. There was no difference in mean water temperature at the two sites, except that water temperature was lower in March at Tateyama Bay when the early stages of reproduction occur (Matsumoto, 1997).

Discussion

Fan shape generally appears to be the result of regular bifurcation of branches, although the results of the present study show that the shape consists of irregular growth and complex bifurcation within a colony. In addition, heterogeneity of growth rate within a community was observed. Previous investigators reported that the growth rate varied both within a colony (Velimirov, 1975; Weinberg & Weinberg, 1979; Brazeau & Lasker, 1992; Castanaro & Lasker, 2003) and within a species (Birkeland, 1974; Velimirov, 1975; Weinberg & Weinberg, 1979; Yoshioka & Yoshioka, 1991; Weinbauer & Velimirov, 1995).

Factors that influence variation in seasonal growth include water temperature (Grigg, 1974; Velimirov, 1975; Coma et al., 1998a), food abundance, and seasonal reproduction (Grigg, 1974). Water temperature and reproduction may explain the seasonal variation in growth in *Melithaea flabellifera*. The independence of branch growth and size may reflect the diversion of energy away from growth toward reproduction. These seasonal variations in parameters affect the growth form of the entire colony.

The highest growth rate occurred in winter when the water temperature was below 20 °C. During this period, growth rate appears to depend on water temperature. While it was also below 20 °C between spring and summer, the growth rate in this period does not appear to depend on water temperature. These observations suggest that growth in *M. flabellifera* is affected by reproduction more than by water temperature. Growth depends on water temperature when reproductive effect is small in winter, while growth is restricted and does not appear dependent on water temperature in the reproductive season in spring to summer.

The loss of branches and compensatory growth occurred accidentally in colonies of all ages and sizes and in all seasons. High growth rates in injured colonies were also observed in other gorgonian species (Weinberg & Weinberg, 1979; Castanaro & Lasker, 2003; Sanchez & Lasker, 2004). It has been reported that octocoral colony size (Grigg, 1972; Velimirov, 1976) and growth rates (Velimirov, 1975; Sebens, 1984; Kim & Lasker, 1997) are affected by water flow. This is likely due to both the beneficial role of water movement in carrying prey (Wainwright & Dillon, 1969; Sebens, 1984; Mistri & Ceccherelli, 1994) and in increasing respiration (Kanwisher & Wainwright, 1967; Sebens, 1987; Mistri & Ceccherelli, 1994). It has been suggested that size limitation occurs due to water flow (Birkeland, 1974; Sebens, 1984). Because fan-shaped gorgonians are passive filter feeders, their morphology is obviously an example of a growth form allowing optimal contact between living colony tissues and water (Theodor & Denizot, 1965; Wainwright & Dillon, 1969; Riedl, 1971; Grigg, 1972; Russo, 1985; Mistri & Ceccherelli, 1993; Weinbauer & Velimirov, 1995). Sebens (1982, 1984) postulated an optimal size for passive suspension feeders based on the ratio between prey capture or energy intake and metabolic cost as a function of water flow.

If suspension feeders grow toward an optimal size for the reasons described above, it seems possible that growth rate would decrease with age. Such a decrease has been reported in many gorgonian species (Grigg, 1974; Velimirov, 1975; Mistri & Ceccherelli, 1993). Although it is not clear whether any changes in growth rate were attributable to the aging of a colony in the present study, there are large risks and few advantages for suspension feeders to grow beyond the optimal size or optimal density of branches. Weinberg & Weinberg (1979) indicated that for each species there is a maximum colony size that is never exceeded. It was also reported that water movement and food supply decrease if the branches of the sponge *Haliclona oculata* collide (Kaandorp, 1991; Kaandorp & de Kluijver, 1992).

Both the previous studies and the results of this study suggest that optimal size and branch density are constraints on indeterminate growth. The compensatory growth of branches to fill in the space of the fan is thought to reflect these constraints. Moreover, it is suggested that positional information (Wolpert, 1969) within the colony is required to constrain and maintain the overall fan shape. Positions in the colony are continuously changing due to the factors such as seasonal variation, non-seasonal variation, and the positive and negative growth of each branch.

Morphological plasticity in the growth form of gorgonians differs from genus to genus (Weinbauer & Velimirov, 1995). Burlando et al. (1991) analyzed the intrinsic growth properties and concluded that there are statistically significant differences between species. It is conceivable from the discussion above that there are variations in the balance between food availability and structural tolerance of the fans of each species or genus (Birkeland, 1974). It appears that this balance is one of the factors regulating the size, shape, or pattern of arborescent organisms.

Conclusions

Both seasonal and non-seasonal heterogeneous growth was identified in *Melithaea flabellifera*. It was confirmed that growth variations are related to seasonal water temperature change when the reproductive effect is small, although growth is restricted and does not depend on water temperature in the reproductive season. It was found that the growth of branches changed within colonies to compensate for vacant spaces in the fan. A focus on the mean growth rate of a colony would not have identified this phenomenon. This compensatory growth affected the non-seasonal heterogeneous growth of each branch within a colony. This suggests that there is a potential optimal density of branches and fan form in this species and that this constrains the entire colony and affects the growth of each branch with high morphological plasticity. Although visually the branching morphology in gorgonian corals appears well regulated, it is not due to simple continuous indeterminate iteration. This means that biological forms maintain their overall regularity following accidental injury by irregular and heterogeneous growth.

Acknowledgments

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Reproduction and the unusual condition of hermaphroditism in *Sarcophyton glaucum* (Octocorallia, Alcyoniidae) in KwaZulu-Natal, South Africa

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Key words: soft coral, reproduction, high latitude reefs

Abstract

The genus *Sarcophyton* is an abundant soft coral on the marginal, high-latitude reefs in KwaZulu-Natal. A 2-year study on reproduction in the most common species, *S. glaucum*, revealed that gametogenesis in male and female colonies takes 9–10 and 16–18 mo, respectively, in this gonochoric, seasonal, broadcast spawner. Gametogenesis and spawning are synchronised in and between colonies, and with other common soft corals on the reefs. Spawning occurs annually in March between full and new moon with the release of spermsacs and the mature oocytes, the smaller oocytes being retained for further development. Thus far, the reproductive attributes of *S. glaucum* are comparable with other studies on the genus. However, the KwaZulu-Natal population of *S. glaucum* manifested the unusual attribute of hermaphroditism in 9% of the colonies, these having Stage I and II spermaries and predominantly Stage III and IV oocytes in their polyps. Small numbers of Stage I and II oocytes indicated that such colonies may be simultaneous hermaphrodites but this could not be confirmed. The complex reproductive strategy and associated measure of hermaphroditism in *S. glaucum* on KwaZulu-Natal reefs are discussed in terms of the species' recruitment success in this marginal, high-latitude environment.

Introduction

Octocorals are predominantly gonochoric, with a few brooding species that are hermaphroditic (Benayahu, 1997), in contrast to the scleractinians in which hermaphroditic spawners are dominant (Benayahu et al., 1990). Early studies on sexual reproduction in octocorals focused mainly on the brooding species, *Alcyonium digitatum* Linnaeus, 1758 (Hartnoll, 1975), and the Xeniidae (Gohar, 1940). More recent studies, however, have shown that octocorals employ diverse reproductive strategies, viz., broadcast spawning as well as internal brooding or external surface brooding (Benayahu et al., 1990). With the exception of the surface brooder *Rhytisma fulvum fulvum* (Forskål, 1775), gonochoric broadcast spawning is the general mode of reproduction in tropical members of the family Alcyoniidae (Benayahu & Loya, 1983; Benayahu et al., 1990; Benayahu, 1997). Brooding is more common among the temperate alcyoniids (MacFadden et al., 2001).

Coral communities in South Africa constitute the southernmost distribution of this fauna on the African coast and fall almost entirely within marine-protected areas (MPAs) in northern KwaZulu-Natal (Schleyer, 2000: Fig. 1). The reefs on which they are found are not true, accretive reefs since corals grow merely as a thin veneer on the limited Pleistocene sandstone substrata in the region

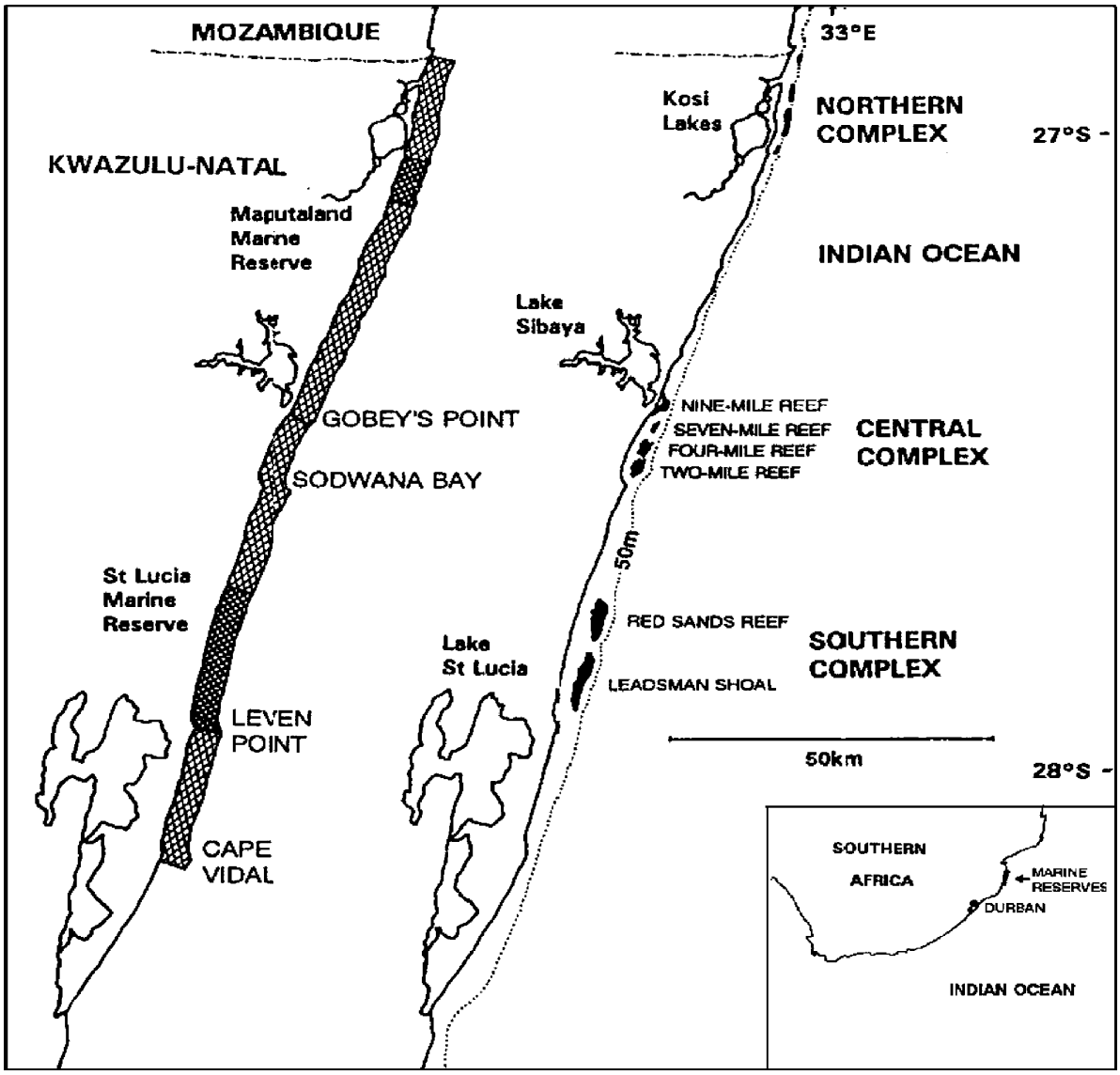

Figure 1. The major coral-inhabited reefs in KwaZulu-Natal fall within reserve (light shading) and sanctuary (dark shading) areas in the Maputaland Marine Reserve north of Gobey's Point and the St Lucia Marine Reserve to the south. These marine protected areas (MPAs) now form part of the Greater St. Lucia Wetland Park.

(Ramsay & Mason, 1990; Ramsay, 1996). As coral reefs, they are thus marginal in nature. Despite this, the coral communities attain a high biodiversity at this latitude on the East African coast and soft coral cover, comprising relatively few species, exceeds that of the more diverse scleractinian cover over much of the reef area (Riegl et al., 1995; Schleyer, 2000).

Intensive research commenced on the reefs in the early 1990s. An early question posed during these studies was whether sexual reproduction occurred in the coral communities in view of their marginal nature. Seven coral species, manifesting the full range of reproductive strategies, were thus examined histologically, and normal reproduction was encountered (Schleyer et al., 1997; Kruger & Schleyer, 1998; Kruger et al., 1998). The results on *Anthelia glauca* Lamarck, 1816, and *Pocillopora verrucosa* (Ellis & Solander, 1786) were published in detail (Kruger & Schleyer, 1998;

Kruger et al., 1998) as representative of many of the octocorals and scleractinians on the reefs. *Sarcophyton glaucum* Quoy and Gaimard, 1883, was also studied, and this paper provides information on the findings on its reproduction and also on the presence of hermaphroditism in the South African material, an attribute that has not been previously encountered in the genus. This would constitute an example of the mixed breeding pattern noted in a few soft corals by Benayahu (1997).

The genus *Sarcophyton* (Octocorallia: Alcyoniidae) is a large taxon with 36 valid species inhabiting coral reefs from the Red Sea and eastern Africa to the western Pacific Ocean (Verseveldt, 1982; Verseveldt & Benayahu, 1983). The colonies are mushroom-shaped with dimorphic polyps, having a capitulum of large autozooids that bear tentacles and gonads, as well as smaller siphonozooids that lack obvious tentacles (Fabricius & Alderslade, 2001). Several species occur in the coral communities at Sodwana Bay (Fig. 1) in KwaZulu-Natal (Benayahu, 1993), of which *S. glaucum* is the most abundant.

Materials and methods

S. glaucum colonies were collected on Nine-mile Reef in the St. Lucia Marine Reserve, KwaZulu-Natal, on the east coast of South Africa (27° 24.9′ S; 32° 43.6′ E; Fig. 1). A full description of the reefs and the local environmental conditions is provided by Schleyer (2000). The colonies were randomly sampled using SCUBA at an average depth of 15 m from September 1991 to November 1994. Monthly sampling was undertaken in 1992 and 1993 to ascertain the reproductive trend followed by this species, after which intensive bimonthly sampling was included during the peak reproductive period (January–March) in 1994. Samples were taken during the weeks of the new moon and full moon during the latter period, thus establishing the various gametogenic stages and the time of spawning based on the disappearance of mature gonads from the samples. Colonies larger than 200 mm in diameter were sampled, but the gonads were checked in 16 colonies of *S. glaucum* of 150 mm diameter. When sampling, a tangential slice (~50 mm thick) was removed with a knife from eight to ten colonies and fixed in 4% formaldehyde in seawater. In the laboratory, the samples were rinsed with freshwater and then preserved in 70% ethyl-alcohol.

Subsamples of each sample (three thin slices, 2 mm thick) were decalcified in formol-nitric solution (Mahoney, 1966), washed, and then transferred to 70% ethyl-alcohol. The gonads of five polyps of each decalcified subsample were dissected for examination under a compound microscope. The number of gonads in each polyp was noted and the diameters of oocytes and spermaries were measured with a micrometer. Histological sections were prepared to ascertain the various gametogenic stages. The material was dehydrated in a graded series of methanol and ethyl-alcohol, cleared in isopropanol and then infiltrated with Paraplast (melting point 57–60 °C) using a Biorad microwave processor. Longitudinal sections of 4–6 μm were cut using a microtome and mounted on glass slides. After staining the sections with Ehrlich's haemalum stain (Drury & Wallington, 1967) and aqueous eosine (Mahoney, 1966), permanent slides were made using mounting medium and coverslips.

The description of the gametogenic stages was based on the classification of Glynn et al. (1991) and the frequency of occurrence of the stages was recorded in five polyps in each subsample. The χ^2 goodness-of-fit formula (Zar, 1974) was used to test for deviations from a 1:1 sex ratio.

Results

Microscopic examination of the fresh material revealed that immature oocytes in *S. glaucum* are opaque and cream-coloured, changing to salmon-pink, orange, or mauve at maturity. The spermaries are more transparent and light cream in colour. After preservation in formal-saline the gonads become orange. The gonads are separately attached to six of the eight mesenteries (four lateral and two ventral) by pedicels continuous with the parental mesogloea and endoderm. Small gonads tended to aggregate in the oral area of the polyp cavity and, as they matured, they were found arranged between more mature gonads borne along the entire length of the mesentery, filling the polyp cavity (Figs 2a and 5a).

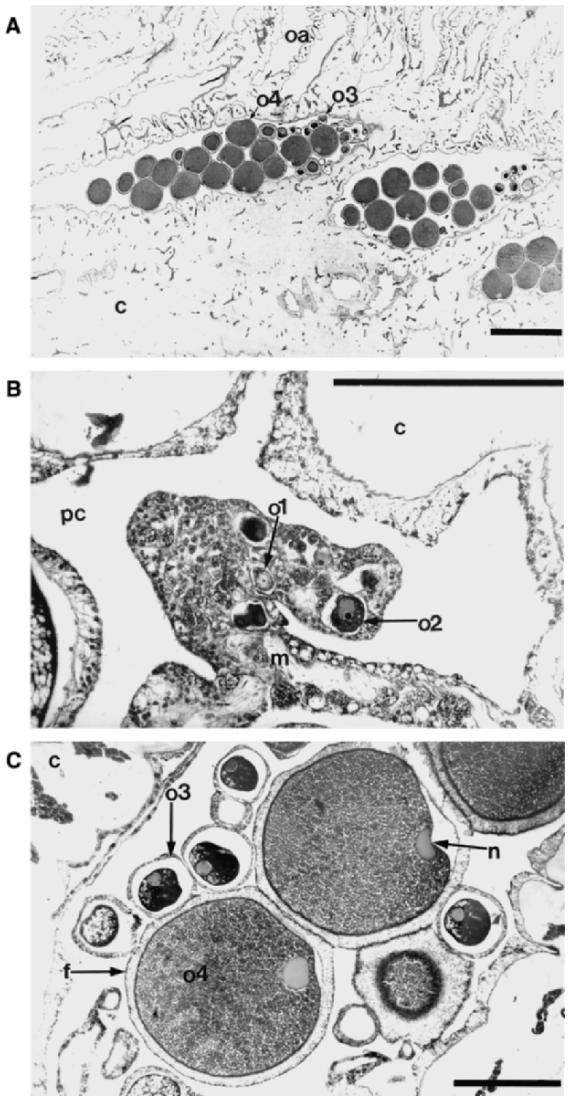

Figure 2. Oocyte development in *S. glaucum*. (A) General view of female polyps showing the oocyte arrangement and the size difference between Stage III and IV oocytes. (B) Stages I and II oocytes embedded in the mesentery. (C) Stages III and IV oocytes in the polyp cavity. Note the indented nucleus in each Stage IV oocyte. c, *coenenchyme*; m, *mesentery*; n, *nucleus;* o1, *primordial oocyte;* o2, *Stage II oocyte;* o3, *Stage III oocyte;* o4, *mature Stage IV oocyte;* oa, *oral area of polyps;* pc, *polyp cavity;* f, *follicular cell layer*). Scale bars = 1000 μm (A) and 250 μm (B and C).

Sex ratio, fecundity, colony size at sexual maturity, and sexual activity of colonies

Of the 276 colonies analysed, 134 were female, 96 were male, 25 bore both oocytes and spermaries in the polyps, and 21 were inactive. Nearly 85% of the polyps analysed in the sexually active colonies were reproductive. The sex ratio did not differ from 1:1 ($\chi^2 = 0.824$; $0.5 > p > 0.25$; d.f. = 1). The sex ratio and the extent of development of the oocytes and spermaries checked in the *S. glaucum* 150 mm in diameter were the same; the collection of material from specimens larger than 200 mm in diameter thus ensured that immature colonies were not sampled. The mean number of oocytes or spermaries per polyp for the year was 43.8 ± 26.5 ($n = 658$) and 95.5 ± 69.9 ($n = 407$), respectively. There was a marked decrease in spermaries per polyp from 99.0 ± 50.6 ($n = 127$) in February to 20.0 ± 10.6 ($n = 15$) in March for the years 1992–1994 combined.

Oogenesis and oocyte development

There were four oogenic stages (Fig. 2) and a fifth atretic stage; these are summarised in Table 1. Oocytes were found in female polyps throughout the year (Fig. 3). The production of small oocytes (Stage I) commenced in November (Fig. 3) and continued for about 6 mo (Fig. 4) without being prominent in any given month. Stage III oocytes were present throughout the year (Fig. 4). There were two oocyte size classes in the polyps from November to March (Fig. 3), with smaller oocytes in the oral area or arranged between the larger oocytes in the polyp cavity. From November to March, the difference in size between the two size classes increased (Fig. 3) as the Stage III oocytes developed into mature Stage IV ova and the Stage III oocytes decreased in number (Fig. 4). The mature oocytes decreased in number or disappeared from the polyps in March, each year leaving mainly Stage III oocytes in the polyps in April (Figs 3 and 4). These again increased gradually in number from March to October, whereafter they were replaced by Stage IV ova as described before (Fig. 3). Atretic Stage V oocytes were only found once in March 1992 (Fig. 4), indicating that the majority of the eggs were spawned.

Spermatogenesis and spermary development

Each fertile male polyp contained spermaries mainly at similar stages of development (Fig. 5); these are summarised in Table 2. During the sam-

Table 1. Oogenic stages in *S. glaucum* collected at Sodwana Bay

Stage	Mean diameter (μm)	*n*	Description
I	14.3 ± 4.0	14	Clusters of primordial oocytes in the mesoglea of the mesentery with large nuclei.
II	48.0 ± 25.4	23	Oocytes separately attached to the mesentery by pedicel. Nuclei centred in the avacuolate ooplasm with conspicuous nucleoli.
III	165.7 ± 52.7	46	Oocytes with vacuoles patchily distributed in the ooplasm and undergoing vitellogenesis.
IV	513.1 ± 67.1	30	Vacuoles evenly distributed throughout the azooxanthellate ooplasm, giving it a fine granular appearance. Nucleus indented and located at periphery of mature oocyte.
V	Disintegrating		Atretic and without defined internal morphology.

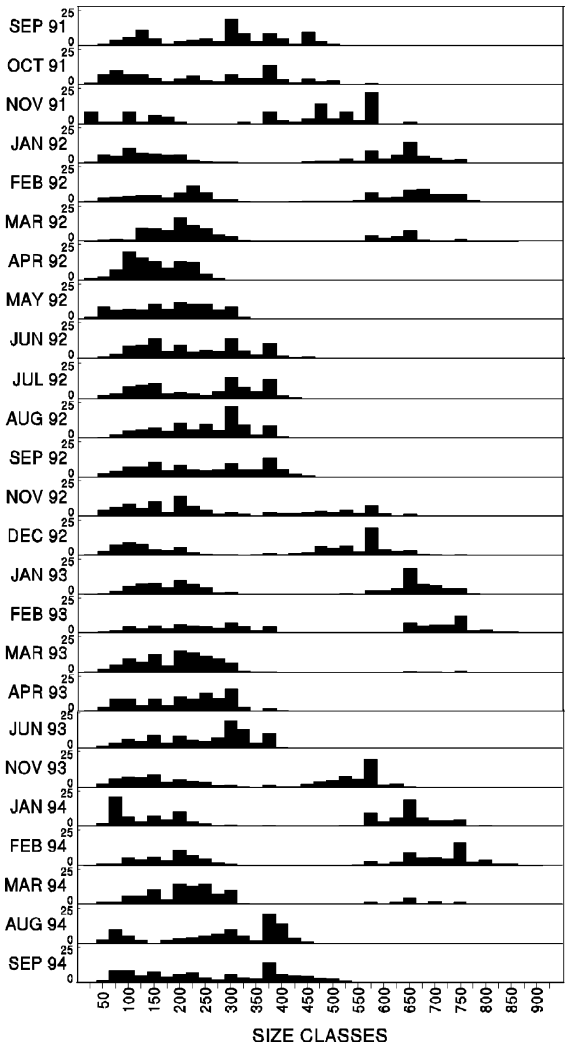

Figure 3. Percentage size frequency of oocytes found in female *S. glaucum* polyps. Size classes are in μm. No samples were collected in omitted months except in October 1992 when no active female colonies were collected.

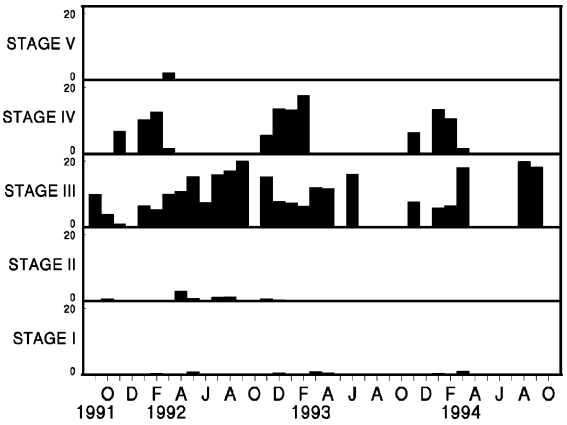

Figure 4. Monthly mean number of oocyte stages found in histological sections of fertile female *S. glaucum* polyps ($n = 402$).

pling period, no fertile male colonies were found after March for some months. Spermaries (Stages I and II) again appeared in June and July (Fig. 6) and enlarged gradually over the ensuing 6 mo until December (Fig. 7). Hereafter, they rapidly developed into Stage III spermaries (Fig. 6), increasing rapidly in size (Fig. 7). They peaked in number in January (Fig. 6) prior to spawning, a sharp decline in the size and number of spermaries per polyp being found in March 1994 when small residual spermaries at the spermatid stage (Stage III) were found (Figs 6 and 7). Mature Stage IV spermaries were found only in February 1994 (Fig. 6).

Hermaphroditism

S. glaucum proved to be mainly gonochoric, although 25 of the 276 colonies examined contained both oocytes and spermaries in their polyps

(Fig. 8). In terms of size distribution, the gonads in these hermaphroditic colonies were similar to female colonies, bearing two size classes of oocyte (Fig. 9) when this occurred. They differed in that the smaller gonads were spermaries, not oocytes. In the months in which only one oocyte size class was to be found in female colonies, the mean diameters of the gonads in the hermaphroditic polyps were largely the same as those found in respective male and female colonies (Fig. 9). An exception occurred in February 1994 when the spermaries in hermaphroditic colonies were smaller than those of mature, pre-spawning size in male colonies (Fig. 10). In terms of development, the spermaries in the hermaphroditic colonies were at Stages I and II and the oocytes at Stages III and IV (Fig. 8a and b) except in September 1992 and February 1993 when a few Stage I and II oocytes were also found.

Lunar phase and spawning

The numbers of the large oocytes and spermaries decreased every year in March (Figs 11 and 12), presumably marking the spawning event. Samples closest to this event were collected on the 28 February 1994 immediately after full moon when large oocytes were still present; these decreased in number before new moon on the 12 March 1994. Spawning, in this case, thus appeared to occur in the period between a full moon and new moon in March. The actual spawning event was not observed. As no brooding of embryos or larvae was found in the colonies, the mature oocytes appeared to be dispersed by broadcast spawning.

Discussion

The gonadal structure and reproductive mode of broadcast spawning in *S. glaucum* are similar at Sodwana Bay (this study) and in the Red Sea at Eilat (Benayahu & Loya, 1986). A sex ratio of 1:1 was found in both studies, with two oocyte size classes developing simultaneously in female polyps for part of the year due to egg retention and maturation for over a year. However, 9.1% of the KwaZulu-Natal colonies contained both oocytes and spermaries, manifesting hermaphroditism, a feature not found in the Red Sea population. Both

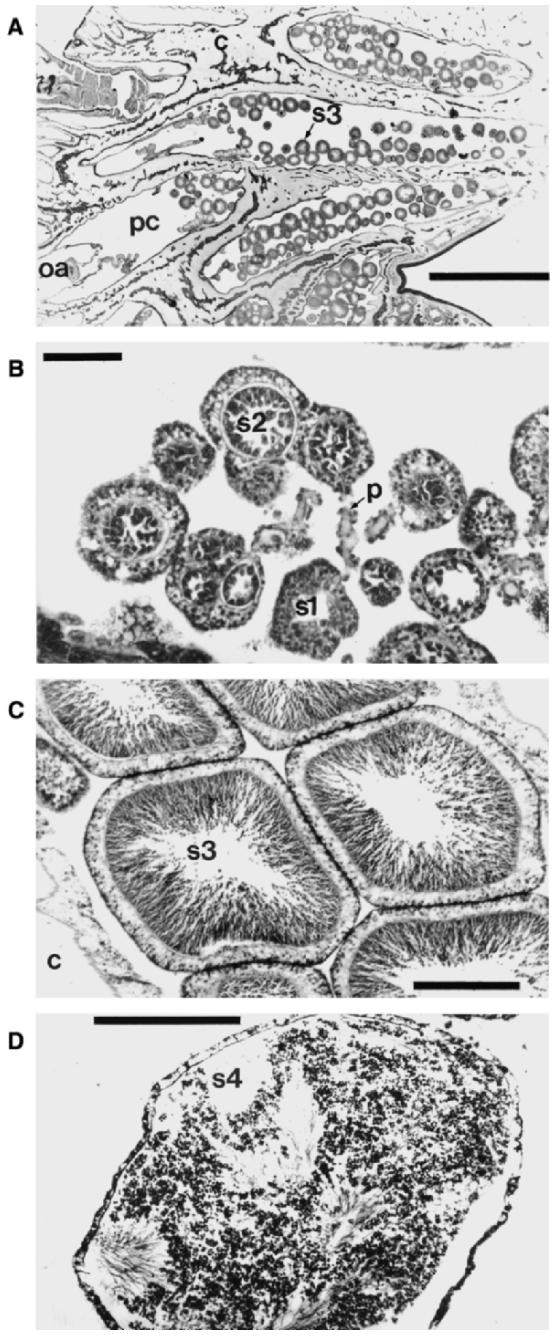

Figure 5. Spermary development in *S. glaucum*. (A) General view of male polyps showing synchronised spermary development. (B) Spermaries with spermatogonia and spermatocytes. (C) Spermaries with spermatids. (D) Mature sperm with tails. c, *coenenchyme;* oa, *oral area of polyp;* p, *pedicel;* pc, *polyp cavity;* s1, *Stage I spermary with spermatogonia;* s2, *Stage II spermaries with spermatocytes;* s3, *Stage III spermaries with spermatids;* s4, *Stage IV spermaries with mature sperm.* Scale bars = 1000 μm (A) and 100 μm (B–D).

Table 2. Spermatogenic stages in *S. glaucum* collected at Sodwana Bay

Stage	Mean diameter (μm)	*n*	Description
I	27.1 ± 7.0	7	Spermatogonia, comprising clusters of interstitial cells in the mesenteric mesogloea, with large nuclei and no apparent cytoplasm.
II	144.9 ± 75.1	32	Spermaries, containing small spermatocytes, connected to the mesenteries by pedicels.
III	186.3 ± 0.25	53	Spermaries with a lumen surrounded by spermatocytes and spermatids, the latter having little cytoplasm and more condensed, darker staining nuclei.
IV	246.1 ± 93.4	10	Mature spermaries containing sperm with dark-staining heads, half the size of spermatids, and lightly stained tails.

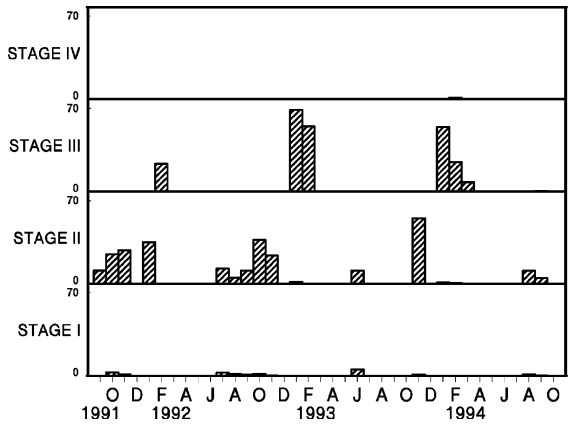

Figure 6. Monthly mean number of spermary stages found in histological sections of fertile male *S. glaucum* polyps ($n = 195$).

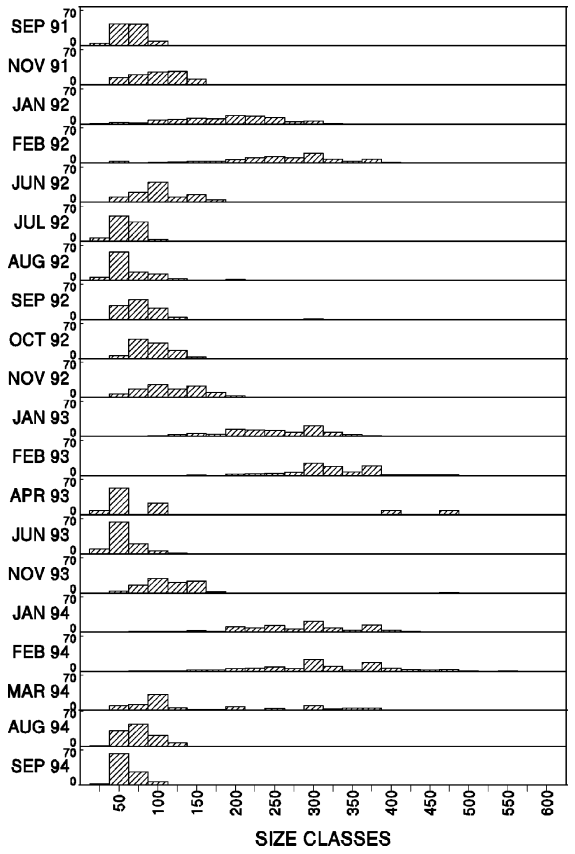

Figure 7. Percentage size frequency of spermaries found in male *S. glaucum* polyps ($n = 8296$). Size classes are in μm. No samples were collected or no active male colonies were found in the samples collected in omitted months.

populations are at the latitudinal extremes of tropical coral distribution, but conditions on the KwaZulu-Natal reefs are marginal (Schleyer, 2000; Schleyer & Celliers, 2003) relative to those found in the sheltered Red Sea environment. This hermaphroditism on the southern African reefs, constituting the first record of a mixed breeding pattern in the genus, may be attributable to these marginal conditions (see further). The oogenic period also proved shorter in KwaZulu-Natal (16–18 mo) than in the Red Sea (22–23 mo), as well as spermary development (KwaZulu-Natal, 9–10 mo; Red Sea, 10–12 mo). The pre-spawning diameter of the spermaries was commensurately smaller in the South African material (250 μm versus 400 μm). There is no apparent reason for these differences, as the temperature regimes are similar at both localities (Schumann & Orren, 1980; IUCN/UNEP, 1988).

Bimonthly sampling at Sodwana Bay provided the approximate time of spawning in March after sea temperatures reach their peak in late February and early March in KwaZulu-Natal (Schleyer, 2000; Celliers & Schleyer, 2002; Schleyer & Celliers, 2003). No embryos or larvae were found in the colonies; fertilization is thus external, as found by Benayahu & Loya (1986) for the same species in the Red Sea. Buoyant eggs, synchronized spawning, the release of prolific gametes, and

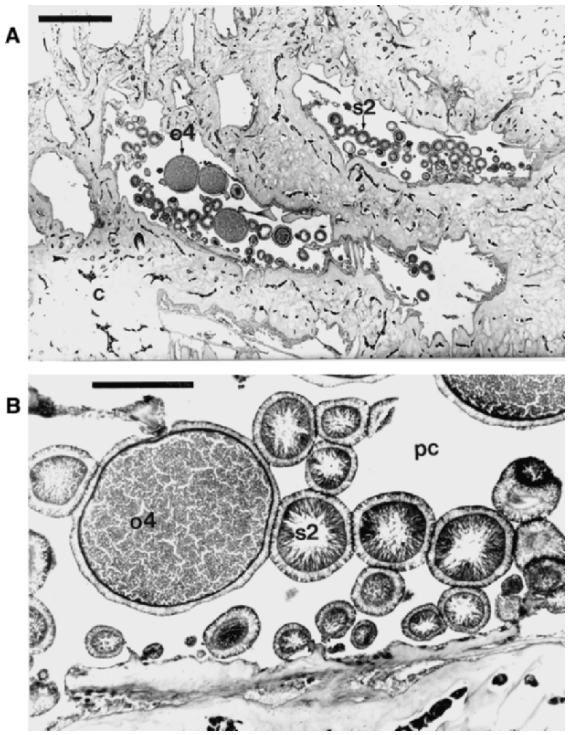

Figure 8. Hermaphroditism in *S. glaucum*. (A) General view of a hermaphroditic polyp showing the difference in size of mature oocytes and late stage II spermaries. (B) Stage IV oocytes and Stage II spermaries in a hermaphrodtic polyp. c, *coenenchyme;* o4, *Stage IV oocyte (mature ovum);* pc, *polyp cavity;* s2, *Stage II spermaries with spermatocytes*. Scale bars = 1000 μm (A) and 250 μm (B).

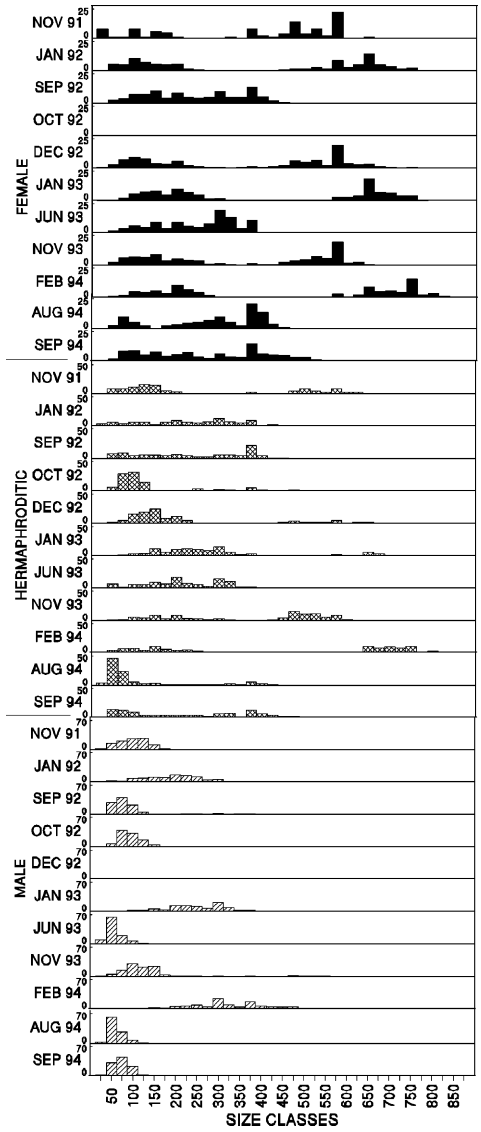

Figure 9. Percentage size frequency of oocytes and spermaries found in male, hermaphroditic, and female *S. glaucum* polyps. Size classes are in μm.

spawning during periods of little water movement can limit the extent of gamete dilution in water, enhancing cross-fertilization (Babcock et al., 1986). Benayahu & Loya (1986) concluded that *S. glaucum* enhances cross-fertilization in the Red Sea by employing all the above, with the release of large numbers of buoyant gametes in a single night. This they suggested improves the survival of *S. glaucum* recruits, a combination of synchronous spawning during low night irradiance with a short period of embryogenesis of 36 h (Benayahu & Loya, 1986) reducing predation on the pelagic eggs and developing larvae. Further protection is gained by synchronising spawning with other members of the coral community, and this was apparent amongst the gonochoric broadcast spawning alcyonaceans studied at Sodwana Bay (Schleyer et al., 1997).

Gamete production was synchronized within the *S. glaucum* colonies on the Sodwana reefs, with the annual production of one gametogenic cycle per polyp. Stage I gametes were, however, produced over an extended period of several months within the population (Figs 4 and 6). Harrison & Wallace (1990) suggest that the cues initiating gametogenesis result in a lower level of synchronization than the cues synchronizing maturation and spawning; there was variability in the onset of

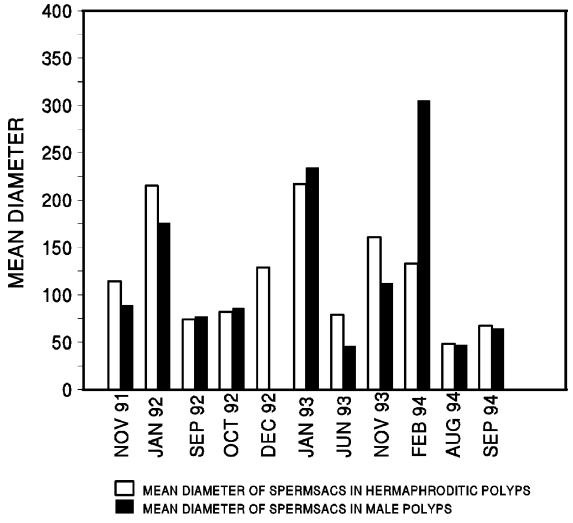

Figure 10. Mean diameter (μm) of spermaries found in hermaphroditic and male *S. glaucum* polyps.

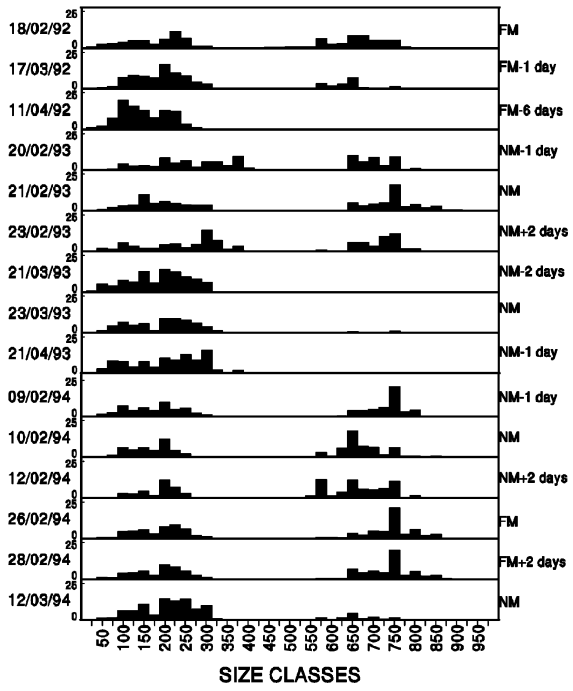

Figure 11. Percentage size frequency of oocytes found in *S. glaucum* polyps during the periods of new moon (NM) and full moon (FM). Size classes are in μm.

gametogenesis within the population but there was increasing synchronicity in maturation towards the spawning event.

Oocyte maturation, the development of oocytes from Stage III to Stage IV, was gradual. Maturation started in November and continued to March (Fig. 4). The mature eggs were large ($\sim$500 μm), a characteristic of soft corals (Benayahu & Loya, 1986). In the case of the Sodwana population, the full period for egg development took up to 17 mo. Synchronization in spermary maturation was greater and Stage II spermaries developed more rapidly into Stage III spermaries in January, with a marked increase in their mean diameter. The fact that mature Stage IV spermaries were found in only 1 mo suggests that their transformation from Stage III to IV was rapid before spawning (Fig. 6).

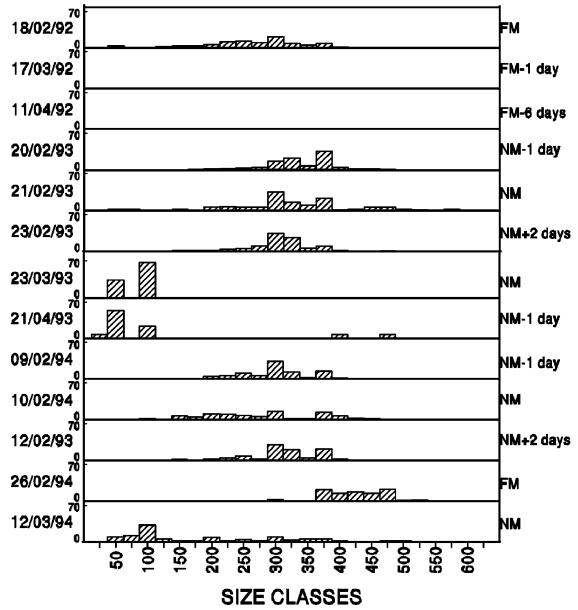

Figure 12. Percentage size frequency of spermaries found in *S. glaucum* polyps during the periods of new moon (NM) and full moon (FM). No reproductively active male colonies were found in samples collected in March and April 1992. Size classes are in μm.

A number of environmental factors undoubtedly influence reproduction in *S. glaucum* on the KwaZulu-Natal reefs. The final maturation of the gametes took place in the austral summer and spawning occurred after sea temperatures attain a peak in late February and early March during waning of the moon in March. These are likely to be the most important regulatory cues for its reproduction and spawning. Tidal flux is likely to be of less influence as the reefs are fairly deep (Schleyer, 2000), as witnessed by the sampling depth of 15 m. It is of interest that Babcock et al. (1994) found mass spawning of stony corals occurs

in the same period on high latitude reefs in Western Australia (28°–29° S) and they also concluded that spawning was synchronized with the lunar cycle.

Colonies of *S. glaucum* as small as 150 mm at Sodwana Bay were mature, as was the rest of the experimental material used in this study. Measurements of the growth of *S. glaucum* in the Red Sea (Benayahu & Loya, 1986) and in the genus on the Great Barrier Reef (Fabricius, 1995) indicated that it is slow-growing, the increase in disk diameter being ≤5 mm *per annum*. Female *S. glaucum* colonies reach sexual maturity in the Red Sea population at an age of 10 years (Benayahu & Loya, 1986), suggesting that a colony of 150 mm in KwaZulu-Natal is at least this age. Despite being slow-growing, the species is fecund; a high percentage of the polyps were reproductively active in the mature Sodwana colonies and produced large numbers of oocytes and spermaries. This, combined with the factors described above, could account for its abundance on the reefs in KwaZulu-Natal (Williams, 1992, 1993). However, Fabricius (1995, 1996) attributes low recruitment to the genus and suggests that their abundance arises from their great persistence and low mortality. Asexual reproduction by fragmentation or colony fission has not been observed in this species but there is some evidence that it may account for locally abundant aggregations in a few other species of *Sarcophyton* (Schleyer, personal observation).

Hermaphroditism or sex change?

As Stages I and II spermaries were found with Stages III and IV oocytes in a small number of hermaphroditic *S. glaucum* colonies, the question arises as to whether this unusual attribute constituted simultaneous or sequential hermaphroditism. A sex change, indicative of sequential hermaphroditism, might occur once the oocytes are spawned, as most of the polyps would then only contain spermaries with no small oocytes for the next year's egg production. Small Stages I and II oocytes were found in a few hermaphroditic polyps in September 1992 and February 1993, and these may have been retained in the polyps during spawning for further development along with new spermaries, indicating true hermaphroditism. Colonies would have to be tagged and monitored to establish which of these was the case. An attempt was made to do so but proved impossible because of tag losses in the turbulent conditions (Schleyer, 2000) found on the reefs.

Why should part of the population be hermaphroditic in this marginal environment? Simultaneous hermaphroditism provides an opportunity for self-fertilization if the gametes are self-fertile (Harrison & Wallace, 1990), reducing reproductive wastage, a potentially important feature in areas with low population densities of the species concerned (Tomlinson, 1966). Yet *S. glaucum* is one of the more common species on the coral reefs in KwaZulu-Natal (Williams, 1992). Furthermore, simultaneous hermaphroditism is prevalent amongst the Scleractinia but is accompanied by the absence or near absence of self-compatibility in those with high dispersal potential (Carlon, 1999). There is thus no evidence in the literature to suggest that the hermaphroditism encountered in the South African *S. glaucum* should be associated with self-fertilization.

However, the presence and relatively high incidence of hermaphroditism in *S. glaucum* may be related to the fact that the KwaZulu-Natal reefs are at the southern limits of coral distribution on the African coast; this awaits further investigation. Geographical variations in reproduction in corals are known, e.g. in *Pocillopora damicornis* (Linnaeus, 1758) which is a broadcast spawner or brooder in different regions (Glynn et al., 1991; Ward, 1992). In all other respects, apart from the hermaphroditism and a slightly shorter duration in gametogenesis, the reproduction of *S. glaucum* at Sodwana Bay proved similar to that found in the Red Sea (Benayahu & Loya, 1986).

Acknowledgments

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The reproductive biology of *Parazoanthus parasiticus* (Hexacorallia: Zoanthidea) in Bermuda★

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Abstract

The zooxanthellate macrocnemic zoanthid *Parazoanthus parasiticus* lives at densities of 3–10 cm^{-2} in the chimney sponge *Callyspongia vaginalis* in Bermuda. It is gonochoric and oviparous. Small oocytes appear in the mesenteries in February–March, grow slowly at first, then increase volume rapidly as seawater temperature passes 27 °C in July. In 1993, oocytes were found to have been shed over 2–3 days around the full moon on 1 September, with inferred small spawnings over the preceding and following full moons. Reproduction had finished by November. In 1999, spawning of azooxanthellate eggs ~250 μm diameter took place on 28 August, two nights after full moon. Spawnings therefore precede those known for four oviparous scleractinians and one gorgonian in Bermudian waters by about a week. The eggs are not shed in bundles and lack zooxanthellae. The unknown embryonic development and larval type in macrocnemic zoanthids is discussed and remains to be resolved by further study with *P. parasiticus*.

Introduction

Parazoanthus parasiticus (Duchassaing & Michelotti) is one of five zooxanthellate sponge-dwelling zoanthid species known from the Caribbean region (West, 1979) but the only one present in Bermudian waters (Sterrer, 1986). In Bermuda, its principal host is *Callyspongia vaginalis* (Lamarck), which forms clumps of clustered cylindrical oscula some 200 mm in height. *P. parasiticus* is a shallow water representative of the Macrocnemina (one of two zoanthid suborders). Unlike Brachycnemina, Macrocnemina are distributed worldwide but most often in deep water: they are difficult to obtain live and have been little studied. The abundance of the sponge containing *P. parasiticus* close to the Bermuda Biological Station (BBSR) provided the opportunity to study the reproduction and spawning periodicity.

Zoanthids, with the known exception only of *Isozoanthus giganteus*, are oviparous, with eggs externally fertilized in the water column (Ryland, 1997). Development in Brachycnemina proceeds to a pelagic, modified planula (Semper's larva), either a zoanthina (family Zoanthidae) or a zoanthella (Sphenopidae), but embryonic development in Macrocnemina is unknown (Ryland et al., 2000). Oviparous hexacorals tend to have annual reproductive cycles with a relatively short spawning period (Harrison & Wallace, 1990; Ryland, 1997; Ryland, 2000). Spawning occurs after nightfall and is often synchronized to the lunar cycle, although water temperature and possibly other ultimate factors are involved. For example, many coral species in the Great Barrier Reef (GBR) spawn in October or November (Babcock et al., 1986), but

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in tropical western Australia the same species spawn in March or April (Simpson, 1991). In the western Atlantic, coral spawning also occurs in relation to moon phase (Szmant, 1991; Wyers et al., 1991). Such regulation of the spawning cycle in marine invertebrates is widespread. One common intertidal zoanthid in the GBR, *Protopalythoa heliodiscus* (Ryland & Lancaster, 2003), spawns almost simultaneously with scleractinian corals ~5 d after the October or November full moon (Ryland & Babcock, 1991). Both the process of spawning and the subsequent development of the zoanthella larva of this species have been described (Babcock & Ryland, 1990), while the incorporation of zooxanthellae into late oocytes ensures vertical transmission of these symbionts (Ryland & Babcock, 1991).

Initially, we studied gametogenesis in *P. parasiticus* to establish the periodicity of the reproductive cycle, the seasonality of spawning, and whether zooxanthellae were vertically transmitted. With spawning clearly linked to moon phase by the histological study, it was then necessary to confirm the predicted spawning *in situ*. Also, given the abundance of *P. parasiticus* in the enclosed Bermudian waters, the longevity of Semper's larvae (Scheltema, 1971), and that the circulation patterns around the Bermuda Rise tend to conserve lagoon plankton at all times of year (Boden, 1951; Boden & Kampa, 1953), it was decided also to search for larvae using plankton nets.

Methods

Samples ~50 mm in length of *Callyspongia vaginalis* containing *Parazoanthus parasiticus* were cut from six marked clumps (A–F) near Castle Cut in Castle Harbour in 5 m depth (Fig. 1). Monthly collections were made from March to August in both 1992 and 1993, but at 2-day intervals from late August through September 1993. One collection was used to determine the density of *P. parasiticus* in the sponge. The samples were fixed in Bouin's fluid, washed, and stored in 70% ethanol. Pieces 5×5 mm were then immersed in 20% hydrofluoric acid for 24 h to remove the siliceous spicules (Langenbruch, 1988) and subsequently dehydrated, cleared in Histoclear, wax embedded, sectioned at 8 μm, and stained in Mallory's Triple

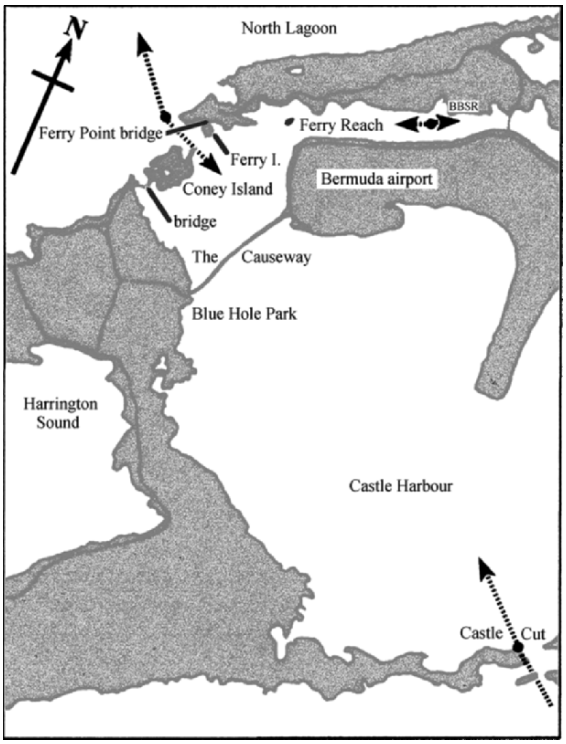

Figure 1. Map of Castle Harbour – Ferry Reach study area in Bermuda, with the direction and strength of ebb/flow tidal currents (from Morris et al., 1977) indicated by arrows.

to display the oocytes and nucleoli. The longest and shortest axes in each of 100 oocytes were measured, using video image analysis, in sections which passed through the germinal vesicle and nucleolus. The former marks the animal pole and the latter (diameter of 8–10 μm) ensured that only one, median section per oocyte was used. The two dimensions were then averaged. Descriptive statistics were performed in Excel and the measurements sorted into classes of 20 μm to construct size frequency histograms. Means of 100 averaged diameters per sample were also converted to volumes to provide a better index of the rate of vitellogenesis. In addition, toward predicted spawning time, a 'gravidity index' was defined which compensated for variations in zooid length: the number of oocytes (nucleoli) present per mm (125 sections) of column. Sea temperatures were not then systematically recorded at BBSR but annual curves have been published (Stephenson & Stephenson, 1972; Morris et al., 1977). A number of spot values were also available from the Bermuda Inshore Water Investigation run by BBSR.

In late summer 1999, direct evidence for spawning was sought in two ways. Two days before full moon, sufficient pieces of *Callyspongia* 'chimney' were collected from large clumps to represent at least three separate male and female *Parazoanthus* clones (safeguarding a possible incompatibility). 'Chimneys' were bagged underwater and then placed over plastic pegs (to anchor them) in running water aquaria at BBSR. At dusk the water flow was reduced, the outflow covered with fine mesh plankton cloth, and the tanks covered with black plastic to exclude extraneous light. This standard method was used by Babcock & Ryland (1990) in the zoanthid *Protopalythoa heliodiscus*. As an alternative, since all zoanthid eggs then known had been buoyant, a *Callyspongia* clump containing female *Parazoanthus*, adjacent to Ferry I. bridge (Fig. 1), was covered during the day by a heavily weighted, 0.5 m mouth diameter plankton net, the bucket of which was buoyed with a float. Male *Parazoanthus* were present below the bridge, about 3 m distant. The net was recovered from the bridge the same night (2200–2300 h), after any spawning would have occurred. As the net had often twisted in the changeable current, the contents were well washed into the bucket which was immediately examined for eggs.

Finally, a 1 m mouth diameter plankton net, 200 μm mesh, was streamed in the cuts north of Castle Harbour to try to capture embryonic or developed larvae from the abundant *P. parasiticus* in the surrounding waters. With tidal amplitude only 0.6–1.0 m, tidal velocities in the channels are much influenced by wind. Published data (Morris et al., 1977) nevertheless suggested that flow under the Coney I. and Ferry I. bridges (Fig. 1) would be sufficient to stream a plankton net. In fact flow did not follow the predicted pattern and had first to be investigated. At times of steady flow around high water, a net weighted with 1–2 kg lead, 5 m overall length, 200 μm mesh, with the bucket buoyed with a float, was deployed for 1 h from both of the bridges (a shorter net would have been more manageable but was not available). The fast flow necessitated firm anchorage for the rope.

Results

Oocyte growth

At the patch reef near Castle Cut (as throughout Castle Harbour) *Callyspongia vaginalis* was the commonest sponge. All the clumps investigated contained *Parazoanthus parasiticus*. The densities of *P. parasiticus* were estimated in each of the marked clumps A–F (Table 1). On histological sectioning it was ascertained that three of the sponges contained exclusively male zoanthids and three exclusively female (gonochorism is usual in Macrocnemina [Ryland, 1997]), suggesting that each was inhabited by a single clone. It is, however, possible that any sponge clump may contain more than one zoanthid clone: this must have been the case in clump D (see below).

Table 1. Description of materials, Castle Harbour, 25 August 1993. Abundances of *Parazoanthus* are based on 10 counts per clump using an area of 1.767 cm^2

Callyspongia vaginalis				*Parazoanthus parasiticus*	
Cluster	Number of tubes	Tube length (range, cm)	Tube diameter (range, cm)	Sex	Mean number (cm^{-2}) ± SD
A	24	15.0–42.0	1.3–3.0	F	5.32 ± 2.76
B	11	2.0–24.5	2.0–2.5	F	6.56 ± 2.34
C	33	4.5–31.0	0.5–2.4	M	9.62 ± 2.46
D	16	4.0–15.0	0.8–2.4	F[a]	2.89 ± 1.29
E	25	1.4–24.0	0.5–2.2	M	5.32 ± 2.12
F	17	6.5–23.5	1.1–2.1	M	7.98 ± 2.07

The densities differ significantly between clumps (ANOVA, $p < 0.001$) but not within them.
[a] There was more than one zoanthid clone in clump D since samples taken after April 1993 were male.

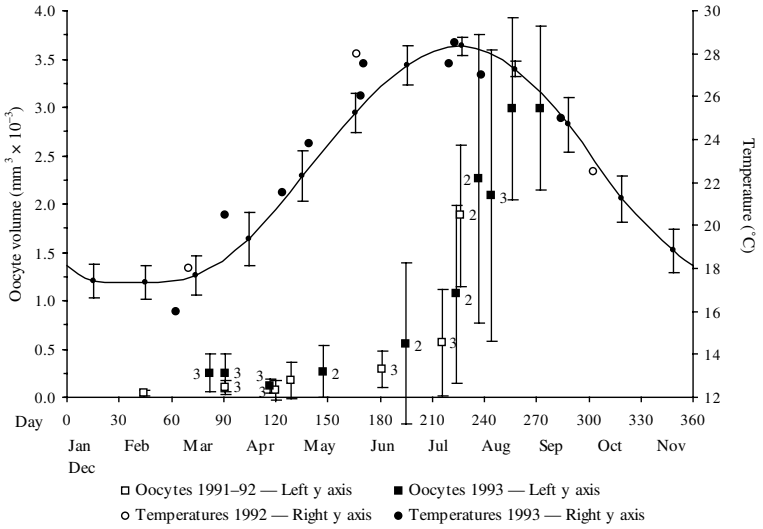

Figure 2. Sample mean oocyte volumes for 1991–1992 (the single point from 1991 indicated) and 1993 (left *y* axis): error bars represent ± 1 SD. When samples have been combined, the number is indicated in the label; $n = 100$ (except for the second sample on day 227, 1991, in which $n = 65$). Sea temperature monthly means (right *y* axis) and ranges (heavy lines) redrawn from Morris et al. (1977) and individual data points, 1992–1993 (provided by Bermuda Inshore Water Investigation).

Oocyte mean volumes have been compared with sea surface temperature (Fig. 2). With no continuous monitoring in 1991–1993, mean sea surface temperatures and ranges have been read from Fig. 6.5 in Morris et al. (1977), though there is no indication to which years they refer. An earlier line, for 1950–1951 (Fig. 5.2 in Stephenson & Stephenson, 1972), matches almost exactly. A few spot points confirm that actual temperatures will have been reasonably close to the means, although the two points for June are 2–3 °C higher. Summer temperatures above the long-term mean were then becoming commoner (Cook et al., 1990). The correlation between oocyte volume (and vitellogenesis) and temperature is striking. Volume remained low from March until June but increased rapidly from July when temperatures would have reached about 27 °C. The breeding cycle is completed at the time of highest temperature. By fortuitous comparison we have one additional value, 0.72×10^{-3} mm^3, in a sample from near Marathon, Florida Keys, 8 March 1993, where temperatures would have been some 7 °C higher than in Bermuda (Fig. 5.2 in Stephenson & Stephenson, 1972).

The error bars in Figure 2 indicate increasing variances as the season progressed, but the means are poor descriptors owing to the frequent non-normality of the sample measurements. More instructive are size frequency distributions, shown sample by sample in Figure 3, remembering that fixation and histological processing cause up to 20% shrinkage. This diagram spans the period from 15 March 1992 until 31 October 1993, and no samples were collected during the 1992–1993 winter period. Oocytes were actually already present in February (Fig. 2) but grew little early in the year. Either few oocytes in any cohort grew or new oocytes were continuously being formed from oogonia (or both): thus towards August, and even in early September, the size range spreads but there is no clear mode, perhaps a reflection of the huge increase in volume required for the egg to mature (Fig. 2). The diagrams for clone A (Fig. 3A) show no clear indication of spawning, as the last three samples show only the disappearance of small oocytes: possibly the shedding of a proportion of the largest oocytes would not be apparent (sampling dates were arbitrary, and not necessarily well timed – the exact relationship with the lunar cycle was, of course, not known). Clone B (Fig. 3B and D), on the other hand, shows a marked reduction in the percentage of large oocytes (>160 μm diam) between the samples of 25 August and 1 September 1993, evidence that spawning occurred during this period, though it

was clearly not complete in the sectioned polyp of 2 September. Only at the very end of the season is there a total absence of small oocytes (Fig. 3A and B). Samples from sponge clump D were originally female (Fig. 3C) but those collected after 23 March 1993 were male.

When it became apparent, from the size of the oocytes, that spawning was imminent, the

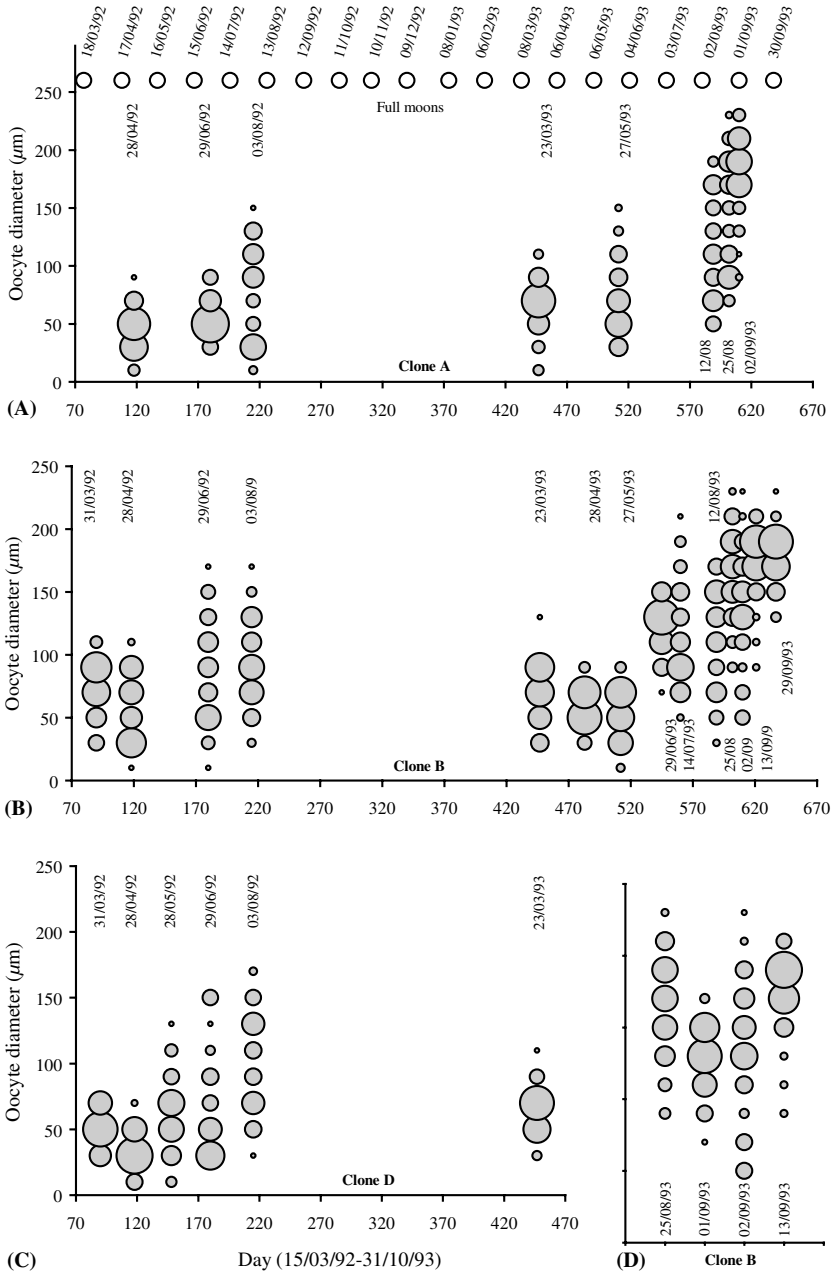

Figure 3. Oogenesis in *Parazoanthus parasiticus*. Bubble histograms of oocyte diameter in samples ($n = 100$) from Castle Harbour, Bermuda, 1992–1993; class intervals are 20 μm and bubbles are centred on the class midpoint; bubble area indicates percentage. Days (abscissae) start from 1 January 1992; day 70 = 15 March. (A) Clone A. (B) Clone B (the sample from 1 September is omitted). (C) Clone D. (D) Clone B, four samples taken over the full moon (1 September 1993) period of spawning; ordinate as C, abscissa intervals arbitrary. Full moons are shown at the top of the figure.

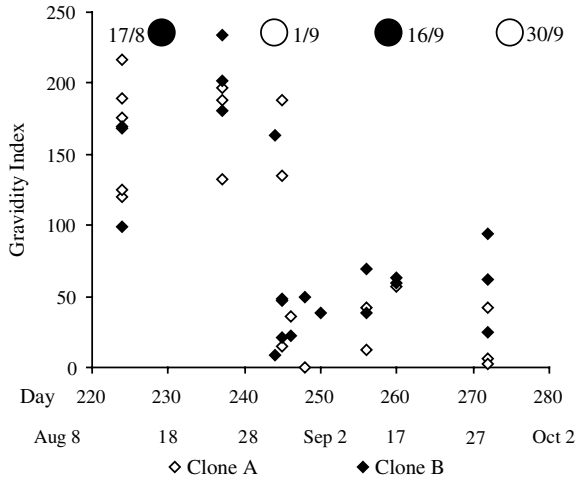

Figure 4. Evidence for spawning in *Parazoanthus parasiticus*. The data plotted are "gravidity index," the number of oocytes per mm zooid column length, in samples of two clones from Castle Harbour, Bermuda, between 8 August and 2 October 1993. Moon phases (new and full) are shown at the top of the figure, the critical full moon falling on 1 September. The sharp fall in gravidity between 1 and 3 September shows that 100–150 eggs per mm column length were spawned over those dates.

frequency of sampling was increased and the 'gravidity index' calculated (Fig. 4). These data show that the gravidity index was high ($\geq$100 mm^{-1}) in polyps of both clones on 12 August and 25 August 1993; on 1 and 2 September 1993 polyps of both clones had some polyps with a high index but in others it was low ($\leq$50 mm^{-1}); and on 3, 5 and 7 September all polyps had a low index (Fig. 4). Full moon was 1 September, so that spawning occurred on two or more nights just before and over full moon. Comparison of the oocyte size distributions in clone B for 14 July and 12 August 1993 (Fig. 3B) shows both a loss of the rather few large oocytes (>150 μm) and the presence of a new cohort of small ones. Together, these suggest that a small spawning took place at or close to the full moon on 2 August. No comparable observations can be made for 1992 since the last samples were taken on 3 August, well before the full moon on 13 August. A few oocytes remained after the 1–2 September 1993 spawning (Fig. 4) and the smaller oocytes grew, resulting in a modal diameter 170–200 μm by 13 September (Fig. 3D) and still apparent on 29 September (Fig. 3B). Unfortunately 29 September was the last sample and the full moon fell on 30th, so spawning at that time can only be inferred. No oocytes were present in November samples (26 November 1992, 19 November 1993).

Observed spawning

BBSR was visited again in 1999 to cover the full moons falling on 28 July and 26 August over which, the 1993 observations demonstrated, spawning of *P. parasiticus* would occur. However, freshly collected *Parazoanthus* in running water aquaria did not spawn on any night, although numerous *Callyspongia* parenchymulae appeared. In one of the tanks, on full moon + 1 day, the water was mixed with an extract of sperm but the zooids are so small that the extract was heavily contaminated with zooxanthellae and sponge cells – this did not initiate spawning. In a different approach, one clump near the Ferry I. bridge was covered with a plankton net each evening, from shortly before sunset to about 2200 h. On 28 August (2 days after full moon), when there was still no spawning, a previously sampled female colony well away from the bridge was checked: it had not spawned. Abundant spawning of *P. parasiticus* eggs ~250 μm diameter into the net then occurred that night. That confirmed the date of spawning in relation to the full moon. The collected eggs were not buoyant when placed in an aquarium and none cleaved; it was assumed that they had not been fertilized. They did not contain zooxanthellae.

Plankton samples

Throughout August numerous hour-long plankton samples were collected by deploying the net from the Coney I. and Ferry I. bridges. Flow, quite unlike the pattern described by Morris et al. (1977), was found to be southerly and maximal around high water (HW), and strong for about 2 h on each side of it. Flow was moderate and northerly around low water (LW), but reduced by a west, northwest or north wind. The high tide period was better, owing to the greater depth of water. Flow was then strong and the net streamed well. Although clogging with scyphomedusae, mangrove leaves, and *Syringodium* was a problem, coral planulae and sponge parenchymulae were obtained but no Semper's larva was ever caught. Occasional zoanthinae, probably of *Zoanthus*

sociatus, and numerous other planulae were found in plankton tows made in the North Lagoon. Thus no larvae possibly attributable to *Parazoanthus parasiticus* have ever been recognized.

Discussion

The oocytes develop at an increasing rate during summer (Fig. 2), leading possibly to three spawnings at monthly intervals between late July/early August and late September/early October, with that in late August/early September the largest. Information on the reproductive periods of other broadcasting Anthozoans in Bermuda is limited to four scleractinian species, *Diploria strigosa*, *D. labyrinthiformis*, *Montastrea cavernosa*, and *M. annularis* (Wyers et al., 1991) and one gorgonian, *Pseudoplexaura porosa* (de Putron, 2003), all of which are reported to undergo one or two spawnings per year over July to September. Spawning of scleractinian species in the Caribbean is similarly restricted to one or two months at the time of and just after maximum seawater temperature, which occurs in August and September at most locations (de Putron, 2003). The reproductive season of the gorgonian *P. porosa* is slightly longer (3–4 months) in the lower Caribbean (Kapela & Lasker, 1999) compared to Bermuda, and such a lengthening of the reproductive season with decreasing latitude also occurs for some scleractinians that reproduce via a brooding reproductive mode (de Putron, 2003).

While the spawning time of *P. parasiticus* approximates to full moon, the actual night(s) range from full −2 days to full +2 days, with some variation between clones (Fig. 4, data for 1 September). This is closer to the full moon than found by Wyers et al. (1991), who recorded synchronous broadcast spawning by *D. strigosa* and the *Montastrea* species 6–8 days after the full moon (within 1 day of the third quarter moon phase). The gorgonian *P. porosa* has also been observed to spawn over these days (de Putron, 2003). Multi-species spawning of a few scleractinian and gorgonian species over the third-quarter moon phase has been observed in Bonaire (de Graaf et al., 1999), and in Panama several gorgonian species mass spawn over the same nights (Brazeau & Lasker, 1989). These are less concentrated events than the spawning of numerous unrelated species over 1–2 nights observed in the GBR (Babcock et al., 1986; Alino & Coll, 1989), where *Protopalythoa heliodiscus* precedes most corals by about 2 days (Ryland & Babcock, 1991). There seems no obvious explanation for *P. parasiticus* spawning several days earlier than the Bermudian corals and gorgonian.

Aspects of reproduction and spawning in *P. parasiticus* are markedly different from those in the only other fully studied zoanthid, the brachycnemic *Protopalythoa heliodiscus*. Apart from being hermaphroditic (a character of the suborder), *Pr. heliodiscus* mature oocytes already contain zooxanthellae and are spawned as egg or egg/sperm bundles (Babcock & Ryland, 1990; Ryland & Babcock, 1991). It is difficult to explain why *Parazoanthus* did not spawn in tanks (though there is a similar problem with soft corals, Y. Benayahu, personal communication). Two possible explanations are: (1) that cutting the *Callyspongia* 'chimneys' releases toxins or other inhibitory chemicals, or (2) that there is some environmental cue missing in a tank. This might be a stimulus from another species or the pressure change of a rising tide (hence linkage to a specific moon phase: on full moon +2 days LW was at 1620 and HW at 2240) – though this would imply a surprising degree of sensitivity since, even on this spring tide, the amplitude was only 0.9 m.

While the failure to obtain fertilized eggs was disappointing, the demonstration of late summer spawning in *Parazoanthus parasiticus* paves the way for additional study. Establishing the pattern of development is an important goal. When Ryland et al. (2000) published their review of the probable development of macrocnemic zoanthids, based on the established restriction of zoanthina larvae to low latitudes (water >18 °C) compared with the ocean-wide distribution of macrocnemic adults, they overlooked the reported occurrence of zoanthinae over the Patagonian shelf (Zamponi, 1982) in water only 10 °C. These outlying occurrences are so far removed from any others that they require some explanation. Although Zamponi (1982) sectioned a larva, he unfortunately chose one with an anomalous pattern of septation, such as is occasionally observed (Ryland et al., 2000): it therefore remains unestablished whether these subantarctic occurrences are of a brachycnemic species of *Zoanthus* or *Isaurus* (which would be

remarkable), or whether they are actually macrocnemic larvae.

Brachycnemic zoanthids are zooxanthellate and have an essentially warm-water distribution comparable to that of scleractinian corals. The most southerly occurring brachycneme is *Zoanthus robustus*, distributed around the southern coasts of Australia (latitudes down to about 39° S) (Shepherd & Thomas, 1982). The approximate temperature ranges along these coasts are: August, 12–15 °C; November, 14–16 °C; February, 16–18 °C; May, 15–17 °C (Tchernia, 1980). These are low enough to suggest that a study of the reproductive biology of *Z. robustus* would be instructive, but still far above the 5–10 °C range over the Patagonian Shelf. It would be remarkable to discover a species of *Isaurus* or *Zoanthus* at such temperatures. The other possibilities are that the larvae are passive immigrants or that they come from indigenous macrocnemes (*Epizoanthus* or *Parazoanthus*). The main ocean current in this region is the cold, north-flowing Falkland Current, but there is an intermittent, south-flowing coastal current conveying water from the warm region off Uruguay and the Rio de la Plata (Arkhipkin, 2001). Given the known longevity of zoanthinae (Scheltema, 1971; Ryland et al., 2000), this origin seems not impossible but, if so, their ability to survive after reaching much colder water was unsuspected.

There seem to be three possible developmental strategies in Macrocnemina: a zoanthina (though the numerous zoanthinae sectioned in the past have never been macrocnemic), a rather undifferentiated (and therefore unrecognized) planktonic planula, or some kind of larva-less development close to the sea bed, as envisaged by Pax & Müller (1962). Sectioning any remaining larvae from Zamponi's collection might resolve the zoanthina possibility but, following the present study, the only realistic way forward is to pursue attempts to obtain and rear eggs of an accessible macrocnemic species, such as *Parazoanthus parasiticus*, in the way that Babcock & Ryland (1990) did for *Protopalythoa heliodiscus*.

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Part VII
Coral Reefs

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Morphological and genetic variation across reef habitats in a broadcast-spawning octocoral

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Abstract

Octocorals, like many other colonial benthic invertebrates, exhibit remarkable levels of morphological variability. The basis for this variability has been largely unexplored. *Plexaura flexuosa* Lamouroux, a common Caribbean octocoral, is found in virtually all reef habitats, and exhibits habitat-related differences in growth rates and fecundity. Population genetic structure and morphological variability in branch and sclerite characters were examined in nine populations from back reef, shallow forereef, and forereef habitats in the Florida Keys. Discriminant analysis of morphological characters correctly classified 89, 88, and 100% of samples from back, shallow fore, and forereef habitats, respectively. Analysis of randomly amplified polymorphic DNA profiles (RAPDs) showed no evidence of genetic differentiation between shallow back, shallow fore, and the majority of forereef colonies. However, there were two distinct genetic groups of forereef colonies. Much of the habitat-related morphological variability among *Plexaura flexuosa* is due to plasticity, which is likely an important feature contributing to the broad distribution of this species. However, there may be two genetic subgroups on forereefs.

Introduction

Although morphological features are the primary means of species identification among colonial anthozoans such as octocorals, for close to a century, workers have commented on the extraordinary morphological and physiological variability exhibited by these taxa (Wood-Jones, 1907). Some studies evaluating the basis for this variability, particularly those using transplant experiments, have pointed to the importance of phenotypic plasticity in response to environmental factors such as light, water flow, and depth (Table 1). Thus, phenotypic plasticity may play an important role in broadening species distributions (Bruno & Edmunds, 1997).

But, as Knowlton (1993) argues, what is interpreted as phenotypic plasticity may sometimes represent ecotypic (genetic) variation (Tureson, 1922). For instance, *Montastraea annularis*, an important Caribbean reef-building coral, was thought to have a broad depth distribution, with depth-related morphotypes (Goreau, 1959) resulting from differences in light availability (Graus & Macintyre, 1976). However, subsequent studies have provided genetic, morphological, and reproductive evidence to separate *M. annularis* into a complex of at least three species (Weil & Knowlton, 1994; Rowan & Knowlton, 1995; Knowlton et al., 1997).

Thus, one challenge for reef ecologists is to assess whether species that are broadly distributed, and for which widespread variation and/or distinct morphotypes are known, represent complexes of sibling species. The Caribbean gorgonian *Plexaura flexuosa* Lamouroux presents a good candidate

Table 1. Studies examining phenotypic plasticity in corals

I. Correlation with habitat or environmental cline
Plasticity present
Agaricia, d (Helmuth et al., 1997)
Alcyonium, f (Sebens, 1984)
Eunicella, f (Velimirov, 1976)
Faviinae, h, d (Wijsman-Best, 1974)
Montastraea, d (Dustan, 1975)
Pocillopora, f (Lesser et al., 1994)
Porites, d (Brakel, 1983)
Plasticity absent
Montastraea (Barnes, 1973)
Platygyra (Miller, 1994)
Porites (Brakel, 1977)
II. Transplant experiments
Plasticity present
Acropora, d, h (Potts, 1978; Oliver et al., 1983)
Briareum, d (West et al., 1993)
Montastraea, d, l, h (Graus & Macintyre, 1976; Foster, 1979; Hudson, 1981)
Pocillopora, f (Lesser et al., 1994)
Sidastrea, h (Foster, 1979)
Turbinaria, d (Willis & Ayre, 1985)
Plasticity absent
Acropora (Potts, 1984)
Pavona (Willis & Ayre, 1985)
Pocillopora (Takabayashi & Hoegh-Guldberg, 1995)
III. Molecular genetic analysis
Plasticity present
Plexaura (this study)
Plasticity absent (i.e. genetic differentiation)
Alcyonium (McFadden, 1983)
Briareum (Brazeau & Harvell, 1994)
Favia (Carlon & Budd, 2002)
Montastraea (Knowlton et al., 1997, Van Veghel & Bak, 1993)
Montipora (Stobart & Benzie, 1994)
Pavona (Willis & Ayre, 1985, Ayre & Willis, 1988)

For each study, genus and type of environmental gradient studied (where appropriate) are given. Abbreviations: f = flow, h = habitat, d = depth, l = light.

species for testing whether phenotypic plasticity or ecotypic variation underlie broad species distributions. *Plexaura flexuosa* is common throughout the Caribbean, inhabiting virtually all reef habitats and a wide depth range (Goldberg, 1973; Kinzie, 1973; Lasker & Coffroth, 1983; Beiring, 1997). Moreover, a recent study found habitat-related differences in growth rates and fecundity (Beiring, 1997; E. Kim et al., submitted). In this study, we examine morphometric and genetic variation among *Plexaura flexuosa* collected across various habitat types.

Materials and methods

Study sites and sampling

Plexaura flexuosa was collected from nine reefs in the upper Florida Keys comprising three habitat types: back reef (≤5 m; Three Sisters, Triangles, and Cannon Patch); shallow forereef (5–6 m; Conch, Molasses, and Pickles) and forereef (15–16 m; Conch, Molasses, and French). Female colonies were used so that egg production also could be measured. Samples were collected approximately 1 week before spawning. On each reef, two primary branches, each ~100 mm long, were taken from colonies taller than 300 mm (the height at which most colonies are reproductive: Beiring, 1997; E. Kim et al., submitted). The sampled colonies were chosen haphazardly from at least two areas of each reef. Branches for morphological analyses were preserved in 10% formalin in seawater and later rinsed in tap water for ~24 h and transferred to 70% ethanol. Branches for DNA analysis were frozen in liquid N_2 and stored at −70 °C until DNA was extracted.

Morphological analysis

An overview of morphometric characters used in this study is given in Table 2. Branch thickness and polyp measurements were made with a binocular dissecting microscope fitted with an eyepiece micrometer. Branch diameter was measured at 20 and 30 mm below the branch tip, and the average was used to estimate surface area (cm^2 per linear cm) of the branch. Polyps between 20 and 30 mm below the tip were counted to determine polyp density (polyps/cm^2). Twenty randomly chosen calyces were cleared of surface sclerites so that calyx diameters and intercalyx distances could be measured.

For each colony sample, the diameters of all eggs in 10 haphazardly selected polyps located

Table 2. Overview of characters examined

Character	Description	Sampling
(a) Colony		
(1) height (cm)	Height from base to tip	1 per colony
(b) Branch		
(2) surface area (cm^2)	Estimated from branch diameters at 2 and 3 cm	1 per colony
(c) Polyps		
(3) polyp density (cm^{-2})	Number of polyps in 1 cm band of branch	1 per colony
(4) calyx diameter (μm)	Diameter of polyp opening	20 per colony
(5) intercalyx distance (μm)	Rim to rim between calyces	20 per colony
(d) Reproductive status*		
(6) fecundity (no. of mature eggs)	Number of eggs ≥500 μm per polyp	10 polyps/colony
(e) Sclerites		
(7–8) club length and width (μm)	Maximum sclerite length and width	20 per colony
(9–10) spindle length and width (μm)	Maximum sclerite length and width	20 per colony
(11–12) capstan length and width (μm)	Maximum sclerite length and width	20 per colony

* Data from Kim et al. (submitted).
Data were collected from a total of 53 colonies. Six were collected from each of three back reefs and each of three forereefs; six were collected from each of two shallow forereefs, and five were collected from a third shallow forereef (Molasses).

between 20 and 30 mm from the branch tip were measured (Beiring, 1997; E. Kim et al., submitted). Fecundity refers to the number of mature eggs per polyp. Previous work (Beiring & Lasker, 2000) had shown that *Plexaura flexuosa* eggs released during spawning are ≥500 μm in diameter; therefore, fecundity was determined as the number of eggs ≥500 μm.

Sclerites were collected from a 10-mm segment between 35 and 45 mm from the branch tip. Sclerites were prepared according to the methods outlined by Bayer (1961). Briefly, using 1–2 drops of bleach (i.e. commercial hypochlorite solution) and a scalpel, the three sclerite layers (surface clubs, middle spindles, inner capstans) were separated from whole dried samples and placed in microfuge tubes with bleach to dissolve any remaining tissue. Sclerites were then rinsed 3× with distilled water and 1× with 70% ethanol, dried 24 h, and mounted on glass slides. Clubs and capstans were measured with the image analyzer Optimas (BioScan Inc., Version 4.10). Images were obtained using a 28× petrographic microscope and a Kodak Megaplus video camera control unit (Model 1.4). Spindles, which were too large to be measured using the image analyzer system, were measured using a binocular dissecting microscope fitted with an eyepiece micrometer. For each sclerite type, maximum length and width of 20 sclerites were measured from each colony. Sclerites were chosen by systematically but blindly moving across the slide and choosing the sclerite closest to the center of each new field of view.

MANOVA was used to determine if there were habitat-related differences in morphology. Discriminant analysis was performed to determine if colonies could be categorized into habitat types on the basis of these morphological characters. All data were tested for, and transformed as needed to meet assumptions of, normality (Kolmogorov test) and homogeneity of variance (Levene test).

Genetic analysis

From each habitat type, 13–14 colonies were examined genetically using Randomly Amplified Polymorphic DNA markers (RAPDs), a technique that has been used successfully in the past in the genetic identification of gorgonians, including *Plexaura flexuosa* (Coffroth et al., 1992; Coffroth & Mulawka, 1995; Coffroth & Lasker, 1998). DNA was extracted from 5 mm pieces of frozen branch by grinding in 0.6 ml 2× CTAB (hexadecyltrimethyl ammonium bromide) buffer (1.4 M NaCl, 20 mM EDTA [ethylenediaminetetraacetic acid], 100 mM Tris-HCl pH 8.0, 2% CTAB, and

0.2% 2-mercaptoethanol), and 0.5 ml phenol (modification of method described in Coffroth et al., 1992; phenol was used to prevent degradation of DNA). After the phenol was removed, 3.6 μl of proteinase K was added to the aqueous phase, the sample was incubated for 1 h at 65 °C, and the extraction was continued according to the methods in Coffroth et al. (1992). RNA was digested by adding 0.5 μl RNase (0.01 μg/μl) and incubating for 30 min at 56 °C. DNA was quantified spectrophotometrically and adjusted to a concentration of 5 ng/μl.

In order to identify primers that produce polymorphisms, nine colonies (one per reef) were screened using 45 primers from University of British Columbia (UBC) Nucleic Acid-Protein Service Unit or from Operon Technologies Inc. Most of the primers produced some polymorphic markers, but none produced markers that were specific to one habitat type. Four primers from UBC that produced polymorphic markers and amplified well (UBC 504, 512, 528, and 554) were chosen and used to amplify DNA from each of the 41 colonies.

As RAPDs can be sensitive to reaction conditions, RAPD profiles were produced as described in Coffroth and Mulawka (1995), where the polymerase chain reaction (PCR) conditions (Mg^{2+}, primer, and DNA template concentration) were optimized to obtain reliable and reproducible patterns in gorgonians. Controls with no DNA were run to check for contamination. One colony was arbitrarily chosen as a positive control for each primer and run twice on each gel. Samples were amplified with one cycle of 2.5 min at 94 °C, 1 min at 35 °C, and 2 min at 72 °C; 45 cycles of 1 min at 94 °C, 1 min at 35 °C, and 2 min at 72 °C; and one cycle of 8 min at 72 °C. Samples were eluted on a 1% Synergel (Diversified Biotech)/0.6% agarose gel for 7–8 h with recirculating 0.5× TBE buffer (0.045 M Tris-borate; 0.001 M EDTA), stained with ethidium bromide, and visualized by UV light. Each gel contained five individuals from each habitat type, two samples of the positive control, and two 123-bp markers.

To verify the reliability of the RAPD markers, RAPD profiles of all samples were replicated at least once. In every case the overall banding pattern in replicate runs was the same, but the intensity of the bands differed in some. Gels were scored conservatively by including only bands that were strong and distinct and repeated in each gel. With the large number, 108, of markers identified in this study, it is likely that any random 'noise' due to non-reproducible markers or PCR artifacts would be overwhelmed by the true variation. Using this approach, RAPDs have been found to be reliable markers (Kjolner et al., 2004).

All colonies were coded for the presence or absence of each polymorphic RAPD marker produced by the primers. These data were used to calculate similarity indices between all pairs of individuals (2 × number bands shared/total number of bands in each individual; Nei & Li, 1979) and average similarities within and between habitat types. RAPD data were also used in a cluster analysis (complete linkage) based on the presence/absence of bands and in an Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992) using the software Arlequin (Schneider et al., 2000) to detect evidence of population differentiation.

Results

Morphological variation

There was significant morphological variability among *Plexaura flexuosa* from different habitat types (Fig. 1). However, there did not appear to be any consistent trends among individual characters across habitat type. For instance, colonies on the shallow forereef had the thickest branches but shared similarities in calyx size with forereef colonies and polyp density with back reef colonies. Colonies in the back and shallow forereefs were taller than forereef colonies, but surface area among shallow fore colonies was higher than in both back and forereef colonies. Shallow fore colonies had the largest surface clubs, but among the smallest spindles.

While individual characters did not vary consistently among habitat types, there was systematic morphological variation among colonies from the three reef types. Discriminant analysis using all of the morphological characters produced two linear functions that correctly categorized 89, 88, and 100% of samples from back, shallow fore, and forereef habitats, respectively (Fig. 2). The functions were highly correlated with habitat type (0.88 and 0.76, respectively: Table 3). The parameters that loaded most heavily on the

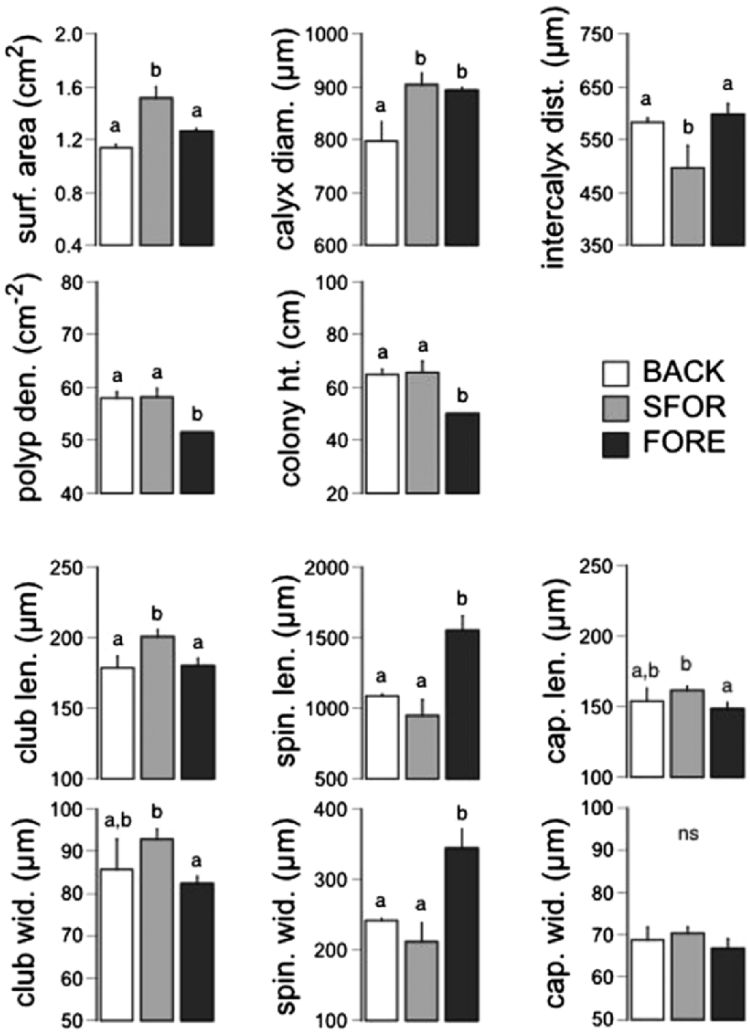

Figure 1. Summary of *Plexaura flexuosa* morphological characters as a function of habitat type. All values represent means ± SE. Letters above bars identify groups with similar means. See Table 2 for character descriptions. BACK = back reef, SFOR = shallow forereef, FORE = forereef.

first function were spindle length and width and colony height. The parameters that loaded most heavily on the second function were branch surface area, calyx diameter, and club length (Table 3).

Genetic variation

The four 10-mer primers (UBC 504, 512, 528, and 554) generated a total of 108 polymorphic RAPD markers across all colonies. The average number of markers per colony was 21 ± 0.58 (mean ± SE). There was no significant difference in the number of markers per individual based on habitat type (ANOVA, $F = 1.21$, $p = 0.31$). No single marker was unique to a habitat, i.e. present in all colonies of one habitat and not present in any other habitat.

Average similarity (Nei & Li, 1979) within shallow back reefs and within shallow forereefs (0.55 and 0.56, respectively) was the same as the average similarity between these two habitats (0.56), indicating no genetic differentiation between the two habitats (Table 4). Average similarity among the forereef colonies was lower

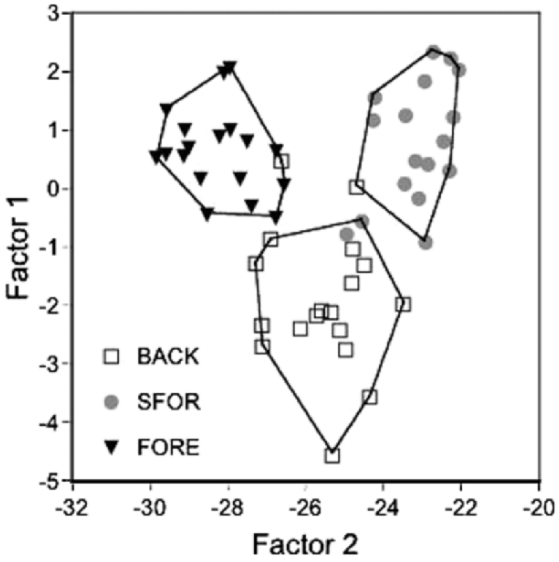

Figure 2. Discriminant analysis of morphological characters (see Fig. 1). Polygons represent groups predicted by the analysis. Abbreviations as for Figure 1.

(0.42), as was similarity between forereefs and the other habitat types (0.46 with shallow back reefs; 0.45 with shallow forereefs).

Complete linkage cluster analysis showed general similarity of colonies from all habitat types, except for one cluster of five forereef colonies (Fig. 3). The group comprised representatives from each of the three forereefs. When this 'outgroup' of five forereef colonies was removed from the similarity matrix, the average similarity among colonies within the forereef habitat rose to 0.52, and the similarity between forereef and the other habitat types rose comparably (Table 4). Average similarity among the five outgroup forereef colonies was also higher (0.57), while average similarity between these colonies and the other groups was low (0.32 for shallow back reefs; 0.29 for shallow forereefs; 0.31 for the remaining forereef colonies). This indicates that the majority of colonies in the forereef habitats were genetically similar to the shallower colonies, while there was a small group of forereef colonies that differed from all the others.

AMOVA revealed significant variation in RAPD profiles among habitats and within reef (Table 5). However, habitat explained only 4.2% of the genetic variation whereas more than 93% was due to differences among colonies within reefs. We tested whether the habitat effect was due to the cluster of five forereef colonies (Fig. 3). When the AMOVA was re-run without them, the habitat effect was no longer detectable ($p = 0.4047$).

Table 3. Canonical correlations and factor loadings for discriminant analysis of *Plexaura flexuosa* morphometric characters ($n = 53$ colonies)

	Function 1	Function 2
Canonical correlations	0.876	0.760
Variables		
Spindle length	−0.460	0.207
Spindle width	−0.352	0.162
Colony height	0.294	−0.202
Fecundity	0.256	0.183
Polyp density	0.232	−0.174
Club width	0.231	0.086
Intercalyx distance	−0.219	−0.159
Capstan length	0.190	0.043
Capstan width	0.142	−0.017
Surface area	0.302	0.622
Calyx diameter	0.007	0.338
Club length	0.254	0.288

Variables are ordered by correlation within function.

Discussion

Corals and other colonial organisms show extraordinary morphological variability. Understanding the basis of this variability is essential for examining species interactions and speciation, assessing changes in community structure (e.g. species diversity), and formulating effective management and conservation plans. Over the last several decades, much effort has focused on differentiating geographic populations, particularly in the context of testing the 'openness' of marine populations (McFadden, 1999; Ayre & Hughes, 2000; Bastidas et al., 2001; Ridgway et al., 2001). These and other studies (Table 1) have shown that both plasticity and genetics contribute to a species' morphological variability, but more importantly that the relative roles of these mechanisms differ among species. Our work suggests the presence of two groups of *Plexaura flexuosa*. The more common group is present in all habitats, is genetically homogeneous (suggesting gene flow among reefs),

Table 4. Average similarities of *Plexaura flexuosa* colony RAPD profiles within and between habitats using Nei and Li (1979) similarity indices

	All colonies	Without deep outgroup
Within habitat		
Shallow Back	0.55 (n = 14)	0.55 (n = 14)
Shallow Fore	0.56 (n = 13)	0.56 (n = 13)
Fore	0.42 (n = 14)	0.52 (n = 9)
Between habitat		
Shallow Back/Shallow Fore	0.56	0.56
Shallow Back/Fore	0.46	0.53
Shallow Fore/Fore	0.45	0.54

and varies phenotypically across habitats. The second group is restricted to the deeper sites and is genetically distinct from the common group.

Habitat-related differences

In *Plexaura flexuosa*, we documented strong habitat-related morphological variation (Fig. 1). The majority of colonies from each habitat type were classified correctly based on their morphology using two canonical functions (Fig. 2), suggesting a strong role of habitat in determining form. The major morphological differences among habitat types include branch thickness, size of spindles and clubs, size and spacing of polyps, and colony height (Fig. 1). Each of these characters could be hypothesized to affect fitness differentially across the three habitats, but no single aspect of the environment appears to correlate with the observed distributions of these characters. For instance, branches were thickest on colonies in the shallow forereefs, where wave action is likely the greatest. This is consistent with observations of thicker branches in sea fans (Grigg, 1972; Velimirov, 1976) and *Briareum asbestinum* (see West et al., 1993) from wave-exposed environments. On the other hand, polyp density was lowest in the forereef colonies (Fig. 1). A decrease in polyp density with depth is common in gorgonians (West et al., 1993) and scleractinian corals (Wijsman-Best, 1974; Davies, 1977; Lasker, 1981; Oliver et al., 1983). This decrease may be due to a decrease in resource availability at greater depths (Kim & Lasker, 1998) or could simply be an epiphenomenon of differential changes in growth rates (i.e. rates of polyp formation and branch extension).

Analysis of RAPD data also revealed habitat-related differences, with colonies on the forereefs genetically distinct from colonies on the back and shallow forereefs. However, this difference appears to be driven by the presence of two groups of colonies on the forereefs (Fig. 3, Table 4). The possibility that the smaller group of forereef colonies is a distinct subpopulation or cryptic species is supported by the absence of mature gonads in these colonies, despite the fact that they were collected shortly before *Plexaura flexuosa* spawning (Beiring, 1997). However, some of the colonies in the larger group of forereef colonies also lacked mature gonads at the time of collection. Reproductive compatibility experiments, transplant studies, and a more thorough genetic survey would help to fully assess the population status of the forereef colonies.

Knowlton & Jackson (1994) have discussed the importance of discerning the population structure of reef organisms for understanding the ecology of reefs as well as their past and future resistance to environmental change. Understanding population structure is also critical for developing management programs. Marine reserves are being established around the world as a means of protecting biodiversity and enhancing stocks of fishes and invertebrates both within and beyond reserve boundaries (Halpern & Warner, 2002; Palumbi, 2002). Protection of species complexes with marked population structure will require careful assessment of candidate populations to ensure that a given refuge contains viable populations of each of the member subpopulations/species.

In the present study, although *Plexaura flexuosa* colonies varied morphologically across habitats, the bulk of the colonies belong to a genetically homogeneous subgroup. This suggests larval mixing across all habitat types and the possibility that any single habitat could, and perhaps does, act as a source for all habitats. Identification of recruitment patterns and larval sources may be necessary to ensure that appropriate areas are chosen for protection, but the genetic data suggest that genetic diversity of *Plexaura flexuosa* can be maintained using any of

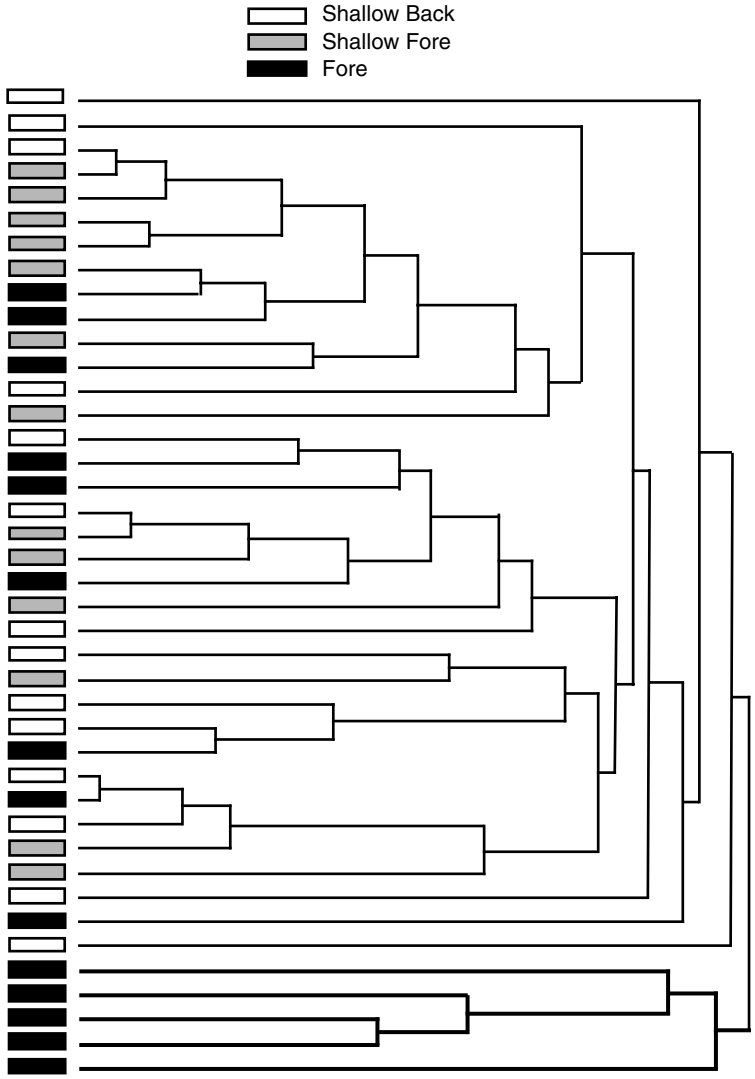

Figure 3. Complete linkage cluster analysis of RAPD data, with cluster of forereef colonies indicated by thicker lines.

Table 5. Analysis of molecular variance of RAPD profile data

Source	d.f.	Sum of squares	Variance	Percent of variation	*P*
Among habitats	2	34.89	0.4565	4.19	0.0127
Among reefs within habitats	6	67.17	0.2117	1.94	0.1906
Within reefs	32	327.6	10.27	93.9	0.0332

the habitats as a source area. In contrast, the genetic distinctness of the forereef morphotype suggests that maintenance of this subpopulation will depend on recruitment from other forereef habitats only.

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Demographic aspects of the soft coral *Sinularia flexibilis* leading to local dominance on coral reefs

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Abstract

We evaluated the role that demography may play in the formation of local aggregations of *Sinularia flexibilis* (Quoy & Gaimard, 1833), a soft coral that commonly dominates inshore coral reefs of the Great Barrier Reef (GBR), Australia. Two populations on inshore reefs of the Palm Islands were censused once a year for 3 years, starting 10 mo after the extensive bleaching mortality in early 1998. Larger colonies became more prevalent over time; mean colony size increasing by 35%, from 276 cm^2 in 1998 to 373 cm^2 in 2000. Growth rates were size dependent, with smaller colonies growing proportionally faster than larger colonies. Change in size relative to initial size indicated an expected mean annual growth of 128 cm^2 for a 50-cm^2 colony. Zero growth was predicted at 532 $\pm$ 21 cm^2, with colonies larger than this likely to undergo fission or shrink. Forty-three percent of colonies were undergoing fission at any time at both localities. Most new colonies were produced by fission (70%, $n = 285$), with the remainder produced by the recruitment of sexually produced larvae (19%) or by colony translocation (11%). The sexual and asexual recruitment rates were 0.24 and 1.0 recruits m^{-2} $year^{-1}$, respectively. Opportunistic recruitment and rapid growth following disturbances are commonly assumed to be the mechanisms leading soft corals to dominate locally. In this study, these mechanisms operated more slowly than expected, with no net change in population size.

Introduction

Despite comprising a large group of species that are represented in most phyla, clonal animals have received little attention compared to aclonal animals or clonal plants (Hughes & Cancino, 1985; Tanner, 2001). Clonal species are morphologically flexible and often have the potential to grow rapidly and attain large sizes. In sessile clonal species, these characteristics may result in the formation of large aggregations. The great capacity of clonal species to survive and recover from partial mortality, and a long life-span confer on them many advantages over aclonal organisms (Hughes & Cancino, 1985; Jackson, 1985). Life history differences between clonal and aclonal organisms lead to major differences in their demographies (Hughes & Jackson, 1980; Hughes, 1984; Tanner, 2001), differences that have not been fully explored in marine organisms.

Soft corals are representative of clonal life histories but relatively few studies have examined demographic aspects of their populations worldwide. On the Great Barrier Reef (GBR), Australia, previous studies were conducted on offshore reefs, and revealed a large variability in the life histories (Dinesen, 1985; Lasker, 1988; Fabricius, 1995; Karlson et al., 1996). Studies of soft corals are lacking from inshore reefs where members of the Alcyoniina and Stolonifera form large

aggregations, locally occupying up to 80% of the total coral cover in shallow waters (Fabricius, 1997). The aim of this study was to obtain demographic data of the common Indo-Pacific soft coral *Sinularia flexibilis*, and to determine which life history characters of this species are important in the formation of aggregations on inshore reefs of the GBR, by repeated observation of colonies within permanent transects.

Methods

Study sites were located on fringing reefs of the Palm Islands, Central Great Barrier Reef. Before 1996, hard and soft coral cover ranged from 30 to 75% on the reefs of these islands depending on habitat, but during 1998 the reefs experienced severe bleaching (Marshall & Baird, 2000; Berkelmans, 2001). Ten months after the onset of bleaching, the mean living cover had decreased by 50% (Bastidas, 2002). One of the study sites was in the channel between Orpheus and Pelorus Islands (18°34′ S, 146°29′ E), and the other between Orpheus and Fantome Islands (18°39′ S, 146°30′ E). These two sites, henceforth referred to as Pelorus and Fantome, are 11 km apart and about 25 km offshore from the mainland.

Twenty belt transects, 5 m long and 1 m wide, were permanently established following the depth contour, five each at 2 m and 5 m, at each site. Transect size was appropriate for accommodating a representative number of colonies given the density at the sites. The starting points of the transects were selected haphazardly. We monitored the transects using still photography once a year in December of 1998, 1999, and 2000. A Nikonos V with 28 mm lens and flash was attached to an aluminium frame, which provided a fixed height of 1.2 m from the reef substratum. Spirit levels attached to the frame helped keep the camera plane parallel to the substratum. Each image covered 1.2 × 0.80 m, which resulted in a photo scale of 1:33 (1 mm in the slide represented 33 mm in the field). Approximately 10 images were taken along each transect. After digitizing the photographs and identifying the colonies (see Bastidas, 2002, for further details), we calculated the number of colonies that had died, appeared as new recruits, or survived from one sampling time to the next in the two time intervals between December 1998 and 1999, and between December 1999 and 2000. Since the fate of colonies at the transect borders could not be reliably determined, we considered only colonies that were completely within the area of the transects on all three censuses. Although this may lead to an underestimate of the colony density, this process was essential to ensure the reliability of the fate of the colonies.

We established five size classes so as to have a roughly equal number of colonies within each class. The size distribution of colonies was compared among sites, depths, and years with a log-linear model. Demographic parameters were estimated from the observation of 1747 colonies, whereas annual growth estimates were based on measurements of 1166 colonies, 589 from 1998 to 1999 time interval (i.e. 448 colonies that survived from 1998 and 141 new colonies in 1999), and 577 from 1999 to 2000 (433 surviving from 1999 and 144 new colonies in 2000). New recruits were assumed to settle soon after the mass spawning in November or December; thus, visible colonies (>5 cm in height) of each census in December were considered to have 1 year of growth.

Table 1. Test of partial associations among Size class (<75, <150, <250, <450, <2100 cm^2), Depth (2, 5 m), Locality (Fantome, Pelorus), and Time (1998, 1999, 2000) as factors affecting the number of colonies of *Sinularia flexibilis*

Effect	Df	Partial χ^2	Probability
Size	4	20.027	0.0005*
Depth	1	320.553	0.0000*
Locality	1	28.336	0.0000*
Time	2	0.070	0.9657
Depth × Size	4	5.465	0.2428
Locality × Size	4	116.534	0.0000*
Time × Size	8	38.391	0.0000*
Locality × Depth	1	307.129	0.0000*
Time × Depth	2	2.353	0.3083
Time × Locality	2	2.698	0.2595
Locality × Depth × Size	4	4.542	0.3376
Time × Depth × Size	8	9.852	0.2755
Time × Locality × Size	8	26.885	0.0007*
Time × Locality × Depth	2	0.339	0.8440
Time × Locality × Depth × Size	8	11.448	0.1776

Table 2. Estimation of colony growth parameters under the conditions explained in the footnote

Parameter	Conditions						
	1	2	3	4	5	6	7
Growth1	148 ± 7	132 ± 7	128 ± 7	133 ± 9	123 ± 11	75 ± 10	68 ± 11
Growth2	−83 ± 12	−79 ± 12	−72 ± 12	−39 ± 18	−96 ± 16	−64 ± 13	−46 ± 15
No growth	492	518	512	594	448	567	595
b	0.77	0.47	0.63	0.54	0.75	−0.25	−0.22
R^2	0.710	0.857	0.915	0.965	0.965	0.949	0.967

Growth1 and Growth2 = annual growth in cm^2 as absolute change in size (±SE) for initial sizes of 50 and 700 cm^2, respectively, No growth = expected size of zero growth in cm^2, *b* = shape of the curve, R^2 = proportion of total variation explained by the regression.
1 – Initial size of new colonies assumed as 0 cm^2;
2 – Initial size of new colonies assumed as 20 cm^2;
3 – As 2, but excluding the 10 largest observed changes in size (5 growths and 5 shrinkages);
4 – As 3, but using only data from the first time interval (1998–1999);
5 – As 3, but using only data from the second time interval (1999–2000);
6 – As 3, but excluding new colonies (sexually and asexually generated);
7 – As 6, but excluding colonies that underwent fission.

To estimate the relationship between the changes in colony size over a year and initial size, we used Francis (1995) non-linear model using the program of Ebert (1999). The parameters from the model (mean annual growth at two specified sizes, and the shape of the curve) were estimated for conditions given in Table 2.

The incidence of asexual reproduction by fission was derived from the photographic record of the transects, and confirmed by observations in the

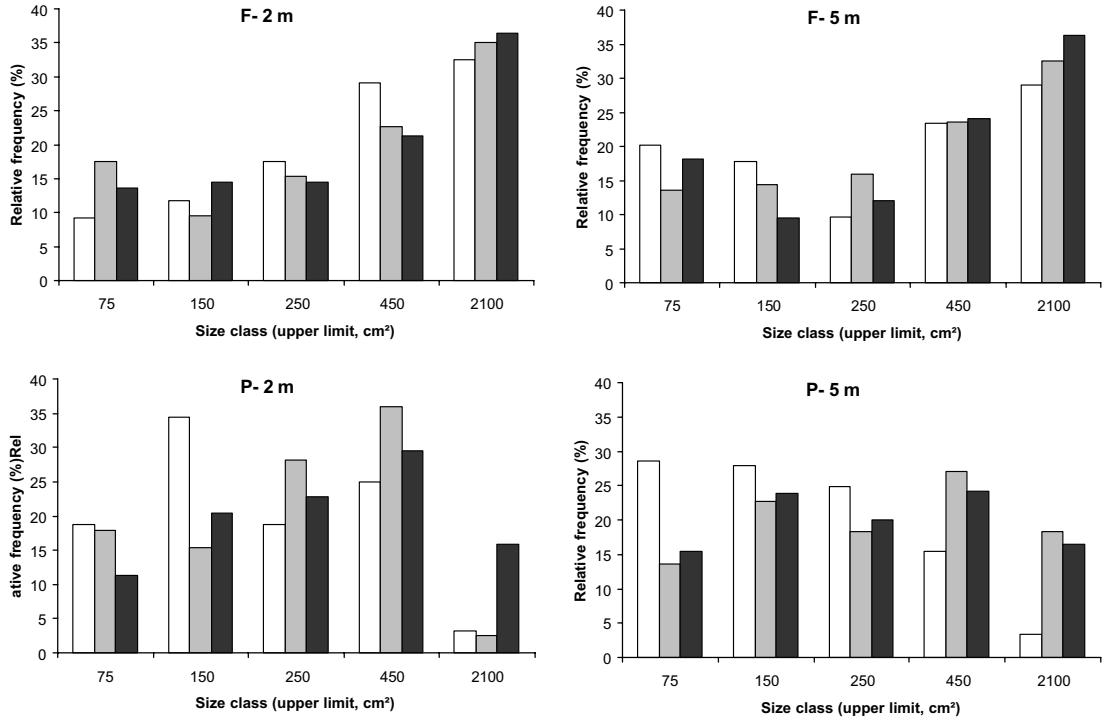

Figure 1. Size class distribution of colonies of *Sinularia flexibilis* at Fantome (F) and Pelorus (P), at 2 and 5 m depth. The white, gray and black bars in each graph represent December 1998, December 1999 and December 2000, respectively.

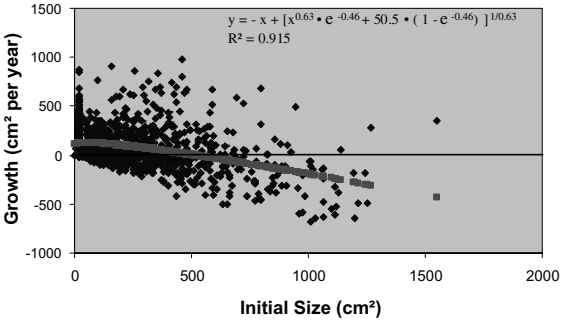

Figure 2. Colony growth function estimated for yearly change in size in relation to initial size at the beginning of that year. Non-linear fits to the model proposed by Francis (1995) include all colonies.

field. Field data from intermediate sampling times (March 1999, August 1999, March 2000, August 2000) were used in addition to the data from December of 1998, 1999, and 2000. It must be noted, however, that the 4118 field observations of colonies are not independent among sampling times for the estimation of the incidence of fission. We used four fission categories: 0 = no sign of fission, 1 = incipient fission, 2 = clear fission, 3 = clear fission in advanced state.

To verify the existence of sexual reproduction, we assessed the presence and size of gonads by examining branch pieces 1–2 cm long under the dissecting microscope. Using this method, we estimated the number of eggs per polyp for three

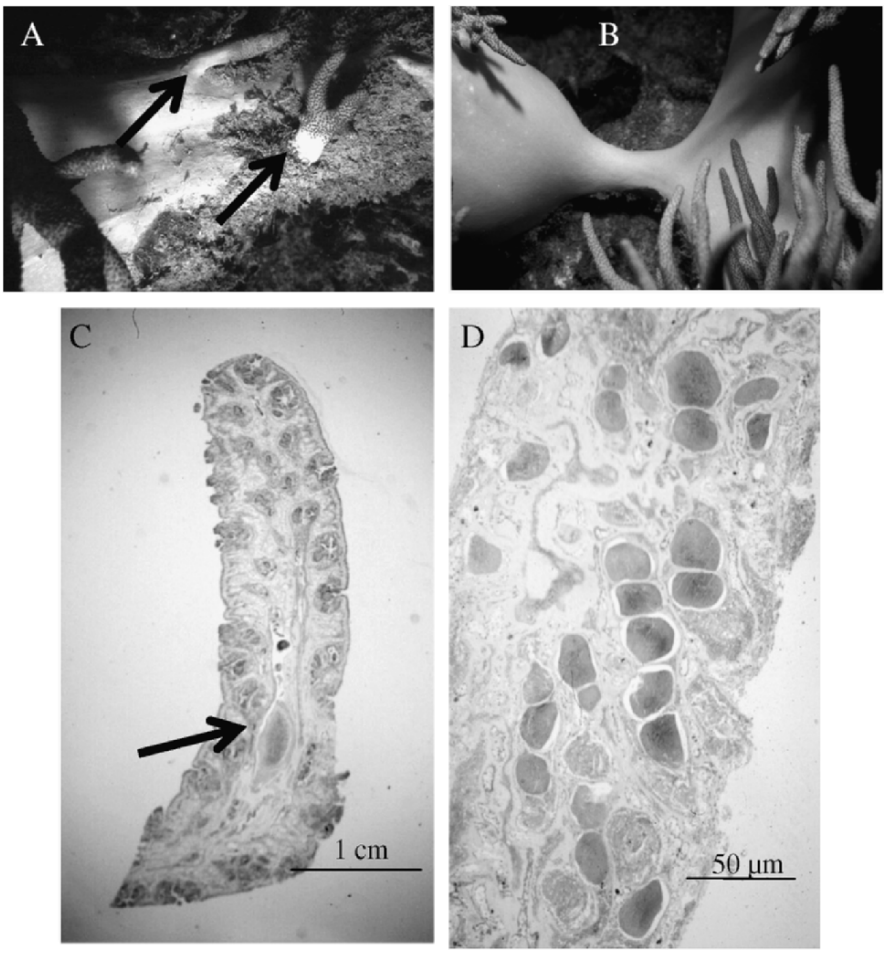

Figure 3. Sinularia flexibilis. (A) Buds at the fission point between two colonies indicated by arrows. (B) Fission potentially producing two daughter colonies; an example of a colony in fission category 3 (=clear fission in advanced state). (C) Arrow points at a mature egg on a branch tip; polyps are visible at the edge of the branch. (D) Spermaries along a colony branch.

or four branches in each of three replicate colonies. We also measured the size of individual eggs in a late stage of maturity (1–3 weeks before mass spawning in December) in 29 colonies comprising the range of colony sizes observed. Between two and six branch tips per colony were histologically prepared using standard embedding in wax and staining with the Mayer's Haematoxylin procedure (Woods & Ellis, 1994). They were serially sectioned and checked for the presence and size of eggs under the microscope.

Results

More than 90% of the *Sinularia flexibilis* colonies in this study were smaller than 800 cm^2, while the maximum size measured was 2018 cm^2. The number of colonies varied significantly in relation to all four factors analyzed (size class, locality, depth, and sampling time; Table 1). The only significant third-order interaction was that the number of colonies in different size classes varied differently through time between the two sites: the size distribution changed clearly through time on Pelorus and less so on Fantome (Fig. 1). At Pelorus, the number of larger colonies increased, while smaller ones decreased through time, with a sharp decline in colonies <100 cm^2 after 1998, particularly at 5 m depth. At both sites, the relative number of large colonies of *S. flexibilis* increased, mainly in the largest size class. As a result, the size of colonies increased in both localities, mainly during the first time interval: mean sizes in 1998, 1999, and 2000 were 276 cm^2 (95%, CI = 248–304 cm^2), 360 cm^2 (95%, CI = 333–388 cm^2), and 373 cm^2 (95%, CI = 342–403 cm^2), respectively.

The change in size as a function of initial colony size showed a curvilinear relation (Fig. 2): while small colonies tended to grow, large colonies often underwent fission, shrank, or remained unchanged. The assumption of an initial size of 20 cm^2 instead of zero improved the amount of variation in growth explained by the regression ($R^2 = 0.857$ versus 0.710, respectively; Table 2). The mean expected growth of 50-cm^2 colonies decreased from 128 ± 7 cm^2 $year^{-1}$ (condition 3) to 75 ± 10 cm^2 $year^{-1}$ when new colonies were excluded (condition 6), indicating that the new colonies had a higher growth rate than other colonies of the same size. Colonies of 532 ± 21 cm^2 were expected to have zero growth, whereas from this size on, size of these colonies was likely to decline through either fission or shrinkage. Thus, it is expected that colonies of 700 cm^2 will reduce on average by 46–72 cm^2 a year. Size reduction is expected to be greater for colonies >700 cm^2, but estimates towards the end of the size distribution are less reliable. As for growth, mortality rate was size-dependent, ranging from 35 to 55% for the smallest size class in different time intervals or sites, and from 8 to 32% for the largest two size classes together.

Sinularia flexibilis is able to reproduce asexually by fission and presumably by the production of buds (Fig. 3). At any time, 43% of colonies were clearly undergoing fission; this estimate was relatively constant throughout the study (41 ± 3.4%), and between the two localities (48 ± 4.8% at Fantome and 42 ± 1% at Pelorus). Fission was mostly binary, starting as an elongation of the colony stem between two or more branches. The height of the stem decreased as the process continued, until two (or more) separate colonies were produced. Colonies were considered to have separated when tissue connecting them appeared not to have any physiological function and seemed to consist mostly of sclerites. The slowness of the fission process, together with the moderately rapid growth up to medium-size colonies, resulted in daughter colonies similar in size to parent colonies.

If 43% of the colonies were undergoing fission, assuming a 50% survivorship for the smallest colonies, and that this process could result in the generation of one daughter colony per year, it would be expected that the 580 colonies observed in 1998 could produce about 125 new colonies by 1999. The observed number of colonies produced by fission was very similar to this estimate (95 in 1998–1999 and 106 in 1999–2000). Thus, colonies clearly undergoing fission were likely to complete this process within a year, and the whole process (from initiation to completion of fission), is likely to take at least 2 year. The field observations indicated that 80% of the colonies undergoing fission were dividing into two colonies (i.e. had the potential to generate one daughter colony), 11% were dividing into three, and the remaining 9% into more than three. The potentiality of colonies

Figure 4. Relative frequency of new colonies recruited by fission, or by sexual means (striped bars) and immigration or translocation (gray bars) into four size classes in the 2 years of the study. Number of colonies is indicated above each column.

to divide, based on these field observations, was in close agreement with the observed production of colonies through fission.

During the study, 76% of the colonies that underwent fission generated one daughter colony (or asexual recruit), 17% produced two, and 7% of colonies produced more than two an up to six asexual recruits. The colonies that resulted from fission accounted for 70% of the 285 newly recruited colonies observed during the study, and they were evenly distributed among the size classes (Fig. 4). The remainder 84 recruits that were not attributable to fission were considered as sexual recruits if they were in the first size class, and immigrants from neighboring transects if they were in larger size classes (Fig. 4).

Buds were found protruding from the stems of colonies, mainly at the colony base (Fig. 3), and at a low frequency (2%) that was similar between localities. This frequency was probably underestimated, as buds are inconspicuous and field observations were time constrained. Buds occurred mostly on dividing colonies, indicating that these two asexual mechanisms are not mutually exclusive. The relative importance of budding as a mechanism of generating new colonies needs further investigation.

S. flexibilis presumably releases its gametes during the coral mass spawning in December (C. Bastidas, personal observation). Not all polyps contained eggs; the number of eggs per polyp varied more among colonies than within a colony (Table 3). Oocytes of two sizes were visible in each mesentery, possibly indicating that oogenesis takes 2 years, which seems common in the Alcyoniidae (Benayahu et al., 1990). Mature eggs were ellipsoid with a mean diameter of 211 ± 5.1 μm ($n = 79$ eggs from four colonies). The range in length of the egg's major axis (170–500 μm) was smaller than that range reported for other *Sinularia* species (900–1150 μm: Benayahu et al., 1990), which probably reflects the smaller polyp size of *S. flexibilis*. Of 129 branches from 29 colonies, nine colonies had eggs and only one had spermaries (Fig. 3). Sexually mature colonies were found in all size classes, but the number of histological

Table 3. Number of eggs per polyp in three colonies of *Sinularia flexibilis* differing in size. Data shown as the range or mean for the subsamples of each colony

Colony (size in cm^2)	No. of subsamples	Polyps/subsample	Eggs/polyp (range)	Eggs/polyp Mean (SD)
1 (157)	3	56–85	0.64–0.83	0.74 (0.096)
2 (236)	4	47–57	0.21–0.33	0.27 (0.056)
3 (628)	3	37–74	0–0.11	0.05 (0.060)

preparations was small. Thus, the fecundity of colonies in the different size class must be further explored. Between 24 and 400 eggs cm^{-2} of branch tip were found in the nine mature female colonies.

Discussion

The soft coral *Sinularia flexibilis* clearly relies primarily on fission for recruitment at the spatial scale investigated; colonies also have a relatively high growth rate at small sizes, yet the population size was relatively constant during the study. The observed changes in size frequency through time supported evidence that these populations had been recently affected by a disturbance, most likely the 1998 bleaching event. A general shift towards larger sizes during the study indicated some population recovery.

The colony size in *S. flexibilis* was determinate, with growth reaching an asymptote at $\sim$500 cm^2, although some colonies reached 2000 cm^2. Most clonal organisms have indeterminate growth, with more exceptions occurring among solitary than among colonial animals (e.g. Grigg, 1974; Hughes & Jackson, 1985). Limits to colony size have been explained by biomechanical constraints on organisms without a rigid supporting structure, and by surface-to-volume limitations for gas exchange (e.g., Cheetham et al., 1980). The shrub-like growth form of *S. flexibilis* colonies may explain apparent growth limits. On the other hand, clones of *S. flexibilis* should be regarded as having virtually no limit to growth through the asexual replication of ramets, as no evidence of senescence was found from the size-specific mortality rates in this study.

Individual growth rates of *S. flexibilis* were inversely related to size, as has been found for other clonal species, including octocorals (e.g., Sebens, 1983; Benayahu & Loya, 1987; Gotelli, 1988; Lasker, 1990; Brazeau & Lasker, 1992; Dahan & Benayahu, 1997), with a few exceptions (e.g. Garrabou, 1999; Cordes et al., 2001). However, absolute growth rates are difficult to compare among species because the variability in growth forms necessitates the use of different types of measurements. A study of encrusting *Sinularia* on mid- and outer-shelf reefs in the GBR showed very little annual change in colony size over 3 years, but for size-dependent growth (Fabricius, 1995). In that study, a 44-cm^2 colony increased by 1.0 ± 0.3 cm $year^{-1}$ in diameter, whereas we expect a colony of *S. flexibilis* of similar size to increase by 7 cm $year^{-1}$. This seven-fold difference in linear growth between species of *Sinularia* on the GBR is one example of contrasting life history and dynamics between congeneric species.

The mortality of *S. flexibilis* colonies was also size-dependent, with the smallest colonies having 2—5× the mortality rate of the largest colonies as is true of most sessile, benthic, clonal invertebrates (e.g., Tanner, 2001). One exception to this generalization is the size-independent susceptibility to some diseases (references in Jackson, 1985). The main source of mortality we identified in *S. flexibilis* is the turnover of colonies when attached to relatively unstable substrata. Sometimes, however, this may serve as a mechanism for the local translocation of colonies. Turnover of colonies is pertinent at the study sites, where tidal currents can reach >35 cm s^{-1} (Fabricius & Domisse, 2000), and where available substrata can be unconsolidated. Coral detachment and abrasion have also been identified as important sources of mortality in other octocorals (Grigg, 1977; Lasker, 1988).

Asexual recruits made a greater contribution to the population of *S. flexibilis* than sexual recruits during the study period. Although at the individual level asexual reproduction is clearly significant, the long-term importance of asexual reproduction at the population level can be difficult to assess. Traditionally it has been suggested that in clonal organism asexual recruitment is more important than sexual recruitment (e.g., Jackson, 1985, and references therein). However, many studies have indicated that this pattern can be highly variable among clonal species (e.g., Harrison & Wallace, 1990; Bastidas et al., 2001). Even when *S. flexibilis* showed more asexual than sexual recruitment, it colonized substrata slowly at the study sites. This contrasts with the traditional assumption that asexual replication is associated with rapid colonization (e.g., Williams, 1975). Many clonal organisms do produce daughter colonies by asexual means at high rates (e.g., Dinesen, 1985), but the production of new colonies can occur just as rapidly by sexual means (e.g., Benayahu & Loya, 1984; Ben-Yosef & Benayahu, 1999).

Sinularia flexibilis reproduces asexually mainly by binary fission that rarely depends on physical fragmentation; instead, initiation of fission appears to be endogenous. The occurrence of colony fission was relatively high (43%). The complete process of division takes 2 years or more to be completed, but in other tropical alcyoniid species studied so far, fission was even slower and less frequent than in *S. flexibilis* (see Benayahu & Loya, 1986; Fabricius, 1995). Fission rates in temperate alcyoniids range from absent in two years of observations, as in *Alcyonium acaule* (see Garrabou, 1999), to rates similar to those in this study, as in *Alcyonium* sp. (see McFadden, 1991). Although *S. flexibilis* showed similar or faster fission rates compared to other studied alcyoniids, the process of division is relatively slow when compared with studied species of the families Nephtheidae and Xeniidae (e.g. Dinesen, 1985; Karlson et al., 1996).

Conclusions

Sinularia flexibilis showed size-dependent growth and mortality. At the spatial scale of this study, and following a bleaching event, asexual recruitment was more important than sexual recruitment. Relatively slow fission rates and high mortality counterbalanced a large proportion of colonies undergoing fission and a rapid growth rate at small sizes, resulting in a stable population size during the study. Based on these results, the formation of large aggregations in this species could take at least a decade.

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Allorecognition and xenorecognition in reef corals: a decade of interactions

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Abstract

Xenorecognition phenomena in coral reefs are expressed by a striking array of morphological and cytological responses. Corals encountering conspecifics further elicit additional repertoires of effector mechanisms, specific to allogeneic challenges. Both inducible sets of antagonistic machineries of allo- and xenoresponses are highly specific. In many cases, they are predictable, reproducible, and reveal the hallmark of coral tissue capacity to distinguish between self and non-self. This essay summarizes a decade (1992–2002) of published results on reef coral immune features. While studies on xenorecognition uncovered the existence of established, non-transitive hierarchies and the importance of antibacterial/cytotoxic compounds secreted by corals, allorecognition assays disclosed the presence of specific and complex non-transitive hierarchies dictated by the expression of a variety of effector mechanisms ('tailored' against different conspecifics), the existence of gradual maturation of alloresponses (important in the formation of natural chimeras), the debatable issue of allorecognition memory, and the appearance of delayed, second sets of alloresponses. A critical evaluation of historecognition reveals that expressed responses in different coral systems are phenotypically matched with counterpart outcomes recorded in the mammalian immune systems. Histocompatibility in corals, as in vertebrates, relies on recognition elements (not yet disclosed on the molecular level) as well as on effector mechanisms.

Introduction

The phylum Cnidaria is among the least morphologically complex metazoan group. Cnidarians differ in ecology and life history traits, but share a body plan comprising two epithelial cell layers with an intermediate acellular gelatinous matrix – the mesoglea. Another ubiquitous feature of the cnidarians is their armature, the stinging capsules (nematocysts), that are discharged following physical or chemical stimulation, causing tissue destruction and cell death. The sessile life styles and growth forms of many members of this phylum promote tissue contacts between closely settled allogeneic conspecifics and xenogeneic partners. These encounters have been extensively analysed in ecological terms, the competition for space (Lang & Chornesky, 1990; Tanner, 1997), or are viewed as the expression of complex somatic recognition events (histocompatibility), ranging from intragenotypic tissue fusion to active cytotoxic rejection (reviewed in Leddy & Green, 1991; Rinkevich, 1996a, b). Leddy & Green (1991) showed that both allogeneic and xenogeneic recognition are common, being effector mechanisms of reactions against antigeneic challenges (Rinkevich, 1996a, b). Many such responses are inducible only upon direct tissue contact (Lang & Chornesky, 1990; Rinkevich et al., 1993; Chadwick-Furman & Rinkevich, 1994; Frank & Rinkevich, 1994; Rinkevich, 1996b; Peach & Hoegh-Guldberg, 1999; and literature therein).

Cnidarian inducible historecognition responses are characterized by a dictation of the effector

mechanism, by employing in many cases specialized structures that are constructed *de novo* or undergo changes upon contact and by the use (not always, i.e. Bigger & Olano, 1993; Griffith, 1997) of distinctive nematocyte populations (Buss, 1990). It is now above dispute that many cnidarians possess highly diverse historecognition systems and effector mechanisms capable of dealing with unlimited number of 'non-self' challenges (Rinkevich, 1996a, b).

Collectively, these historecognition systems have the capacity to identify self cells from non-self entities. Such discrimination is, in principle, exerted by recognizing either the presence or absence of non-self attributes or by detecting the presence or absence of self molecules (Neigel, 1988; Rinkevich, 1996b). Both laboratory and field experiments have further demonstrated that the outcome of this historecognition and the expressed effector mechanisms may result in the demise of interacting partners or in the opposite outcome of prolonged coexistence.

This review of the last decade (1992–2002) of literature dealing with allorecognition and xenorecognition in reef corals is oriented toward the examination of major outcomes in innate immunity. Contrary to other fields of immunity where availability of methods for studying genome-wide expression profiles has led to impressive achievements, in corals there is no accepted synthesis of what historecognition is or does. The number of studies published in peer-reviewed journals on coral defence mechanisms (historecognition and antibacterial properties) during this decade, including abstracts from scientific meetings, is less than four score, which further impedes progress in the field. This does not abrogate the need for critical examination of what is known in an attempt to create a unified conceptual framework for coral self/non-self recognition systems.

Xenorecognition – The art of engagement with foreign attributes

Suites of xenorecognition responses were documented during this period in field experiments on scleractinian corals (Rinkevich et al., 1993; Tanner, 1993, 1995, 1997; Genin et al., 1994; Bruno & Witman, 1996; Van Veghel et al., 1996; Peach & Hoegh-Gouldberg, 1997; Abelson & Loya, 1999; Aerts, 2000; Ferriz-Dominguez & Horta-Puga, 2001) and on soft corals (Alino et al., 1992; Rinkevich et al., 1993; Griffith, 1997). The research over the last decade further emphasizes the potential complexity of the effector mechanisms in xenorecognition interactions between reef organisms. Each of the responses displayed by interacting colonies has distinct consequences on several life history patterns such as growth rates (Aerts, 2000), survivorship (Rinkevich et al., 1993; Abelson & Loya, 1999), and fitness (Tanner, 1997). Results also revealed that the histocompatibility responses are subject to environmental regulations, such as water flow and temperature (Genin et al., 1994) or variable 'environmental' factors (Alino et al., 1992) that may modify their expression. Other regulators of xenogeneic interactions were found to be related to morphogenetic parameters such as the size of the neighbouring partner (Griffith, 1997), and quantity and length of mesenterial filaments (Van Veghal et al., 1996; Peach & Hoegh-Guldberg, 1999).

Studies have also been done examing hierarchies in xenogeneic encounters. Contrary to past results (e.g. Lang, 1973; Cope, 1981; Bak et al., 1982), recent studies confirm the existence of non-transitive hierarchies between different reef dwelling species (e.g. Rinkevich et al., 1993; Tanner, 1993; Abelson & Loya, 1999). This leads to suggestions for the possible ecological role of these responses in shaping coral community structure, using the notion of 'competition' over the term and rationale of cellular xenorecognition (Karlson & Hurd, 1993; Rinkevich et al., 1993; Tanner, 1993; Rinkevich, 1996a, b). Considering xenoreactivities as a reflection of ecological competition, however, reveals only the final outcome of histocompatibility processes, the expression of effector mechanisms, but ignores self/non-self recognition pathways.

It is well documented (Lang & Chornesky, 1990) that colonial reef corals elicit a wide repertoire of effector mechanisms as a response to xenogeneic contacts. Recent studies (Rinkevich et al., 1993; Tanner, 1993; Griffith, 1997) further demonstrate that single colonies may present simultaneously or separately different effector mechanisms, revealing the capacity for 'non-self recognition' pattern over 'self recognition'

(Rinkevich, 1996b). Resolving the underlying biochemical, cellular, or molecular components of the mechanisms involved will require use of modern molecular and biochemical tools not available yet.

Unlike in other invertebrates, such as insects (Jasper & Bohmann, 2002), cnidarian historecognition responses are not based on recognition of common microbial structures such as lipopolysaccharides or peptidoglycans. Although it has not been carefully investigated, no study has revealed opsonization and melanization to kill or isolate pathogens, or any massive mounting of antimicrobial peptides in response to infection (pathogen-induced gene expression). The studies have, however, revealed the constant engagements of 'chemical warfare' against microbes and the continuous secretion of antibacterial compounds (Kim, 1994; Koh, 1997; Wilsanand et al., 1999). Cytotoxic compounds found in secreted mucus sheets (Ding et al., 1994) probably serve as the effector arms against eurokaryotic and prokaryotic invaders, but do not play a role in histocompatibility.

Allorecognition – The realm of discriminating conspecifics

From the traditional ecological point of view, allorecognition in colonial cnidarians may be treated in the context of aggressive interactions, in which one partner of an interacting pair damages its confrere, sometimes leading to its death (reviewed in Rinkevich, 1996a). The allorecognition phenomena, however, as recorded from assays on reef cnidarians, are more than just agonistic encounters. These allorecognition systems are characterized by very precise and specific recognition events, are highly polymorphic, are capable of detecting fine differences even among kin organisms, express arrays of genotype specific effector mechanisms, and exhibit a variety of cellular-morphological characteristics. Studies on allorecognition in reef corals during the last decade have further revealed the omnipresence of this phenomenon in nature.

One of the often-posed issues is the transitivity among interacting conspecifics. This is tested through allorecognition assays tailored to the tested species (Chadwick-Furman & Rinkevich, 1994; Frank & Rinkevich, 1994; Rinkevich et al., 1994). These studies have demonstrated the existence of non-transitive hierarchies in which the most dominant colony in a panel of assays could be subordinate or equal in aggression to the inferior colony. Thus, networks of hierarchies were established among genotypes engaged in allorecognition responses. Moreover, interacting colonies could be clustered into several distinct groups according to their allorecognition responses (Rinkevich, 1996b). A study on the coral *Stylophora pistillata* from Eilat, the Red Sea (Chadwick-Furman & Rinkevich, 1994), in which all pairwise combinations were established, elucidated three allorecognition groups (designated I, II, and III) among nine interacting genotypes. Within each group, colonies overgrew each other in a linear hierarchy pattern. Between groups, they either rejected or overgrew each other; each of the four Group I colonies engaging in non-fusion/overgrowth interactions with Group III and unilaterally rejecting each of the three colonies of Group II. Interactions between Groups II and III were only rejections, and directionality depended on allogeneic partner type. This complicated pattern of incompatibility in *S. pistillata* cannot be explained by the concept of simple 'self recognition' since incompatibility seems to be coded as a series of discrete alternatives resulting from complex genetic elements of the partners. Since all allogeneic responses in *S. pistillata* are highly reproducible (Rinkevich & Loya, 1983; Chadwick-Furman & Rinkevich, 1994), types of responses may not be considered as a causative outcome of external biological or physical parameters.

A detailed historecognition test on the hydrocoral *Millepora dichotoma* revealed additional facets of allorecognition (Frank & Rinkevich, 1994). In 42 allogeneic combinations using 10 colonies, reproducible unilateral tissue and skeleton overgrowth was recorded. These events developed within 10 week and advanced as much as 20 mm. However, they were not the end-product of non-self recognition. Four additional types of secondary responses were later observed among most incompatible combinations: reversals in overgrowth directionality, tissue necroses, standoffs, and abnormal growth. Secondary responses within a set of replicates of most allogeneic combinations were characterized by high variability in

type and intensity. Based on the outcomes of the primary overgrowths, a complex non-transitive hierarchy was constructed for this set of colonies. All isografts and three allogeneic combinations fused within 3 week. Fusion pattern among the three allogeneic combinations was non-transitive. One *M. dichotoma* colony repeatedly fused with two others (four assays each) but these two colonies not only did not fuse with each other, but one repeatedly overgrew its confrére. In the third, the most superior and the most inferior colonies of the 10 fused in all assays.

By contrast, allorecognition responses in the soft coral *Parerythropodium fulvum fulvum* revealed reduced polymorphic responses (Frank et al., 1996). All possible pairwise combination assays ($n = 76$) within two groups of five colonies each, and one group of three colonies were set up *in situ*, each with 2–4 replicates. While isogeneic encounters always resulted in complete tissue fusion, only two types of non-specific allogeneic responses were documented. In the first, interacting allografts retreated from each other a few days following direct contact. In several assays, the colonies repeatedly grew into contact and retreated again. The second allogeneic response was unilateral or reciprocal tissue overgrowth, in which one colony overgrew the conspecific partner by several mm then stopped, and the underlying tissue of the overgrown partner died. No cytotoxicity was observed in allogeneic contacts either in growing parts or when assays were established between cut surface areas. Repeated assays of the same pair-combination were not consistent in terms of type and directionality of responses. This effector arm is therefore restricted only to the level of self-discrimination.

One of the fundamental issues in mainstream immunology, the ontogeny of immune responses, has been studied during the last decade in scleractinian corals (Frank et al., 1997; Hidaka et al., 1997; Hellberg & Taylor, 2002) and in soft corals (Barki et al., 2002). Frank et al. (1997) and Barki et al. (2002) demonstrated for the first time that there is a window in ontogeny, before the allorecognition system matures, when newly settled polyps can fuse, but allogeneic fusion cannot occur at later stages of development. Adult colonies of the reef-building coral *Stylophora pistillata* discriminate precisely between 'self' and 'non-self' attributes, and respond selectively against specific allogeneic challenges. Frank et al. (1997) studied the ontogeny of these allospecific responses on newly settled polyps by establishing allogeneic contacts within groups of siblings or unrelated offspring, observing interactions for up to 8 months. Three types of responses, depending on the age of the interacting partners, were documented. The first was tissue fusion and formation of a stable chimera in partners less than 2 months old. The second was by contacts of 2–4-month-old partners, which started with tissue fusion and followed by separation between chimera partners when the older partner reached the age of 4 months. The third type, the histoincompatibility responses documented in allogeneic interactions of adult colonies (Rinkevich & Loya, 1983; Chadwick-Furman & Rinkevich, 1994; Rinkevich, 1996b), was recorded in all allogeneic encounters with partners over the age of 4 months. Maturation of allorecognition in this species was therefore achieved in 4 months, through three chronological stages. Combinations of siblings or genetically unrelated partners did not affect the results. In a follow-up study, Barki et al. (2002) found that tissue fusion between soft coral allogeneic partners occurs only between young colonies. The lack of an active historecognition system in the early stages of ontogeny in the scleractinian and soft corals studied is probably universal (Bavestrello & Cerrano, 1992; Hidaka et al., 1997; Hellberg & Taylor, 2002).

Chimerism is not beneficial to either partner. Barki et al. (2002), for example, showed that chimeric partners detached, or chimerism resulted in the death or resorption of one or more partners. Long-term observation of these chimeras (to 450 days) also documented slower growth and growth-retarding disorders such as disruption of the structural pattern of polyp budding and polyp configuration. Chimeras are likely to be less suited to field conditions than genetically homogeneous individuals, raising the ecological–evolutionary question of why coral chimeras arose. Juvenile cnidarian chimerism probably represents a case in which ontogenetic allorecognition is not infallible, promoted by gregarious settlement of larvae that is characteristic of many cnidarians (Bavestrello & Cerrano, 1992; Frank et al., 1997: Barki et al., 2002). Allogeneic fusion is probably a primitive

phenomenon: it is recorded in tabulate and ramose corals from the Silurian and late Jurassic periods (Noble & Lee, 1991; Helm & Schülke, 2000).

Another aspect of coral allorecognition studied is memory (Salter-Cid & Bigger, 1991; Frank & Rinkevich, 2001). The exhibition of alloimmune memory, which is derived from selective amplifications of surviving antigen-specific lymphocyte clones, is one of the fundamental characteristics of the vertebrate immune system, enabling an organism to benefit from past experience with foreign antigens. In many mammals, an enhanced secondary immune response to a previously encountered antigen may last a lifetime, years after the primary challenge (cited in Rinkevich, 1996a). Immunological phenomena in other groups of organisms, especially in invertebrates, may be misinterpreted by applying concepts that such as immunological memory that were defined primarily using mammalian models (Kaattari, 1994).

A study on histocompatibility in the gorgonian coral *Swiftia exerta* revealed that all autografts fused while all allografts underwent rejection (Salter-Cid & Bigger, 1991) characterized by rapid and progressive bleaching, necrosis, and loss of tissue in the immediate contact area. Initial reactions required 7–9 days to produce 1 mm of necrosis, but after a resting period, a second contact in a new tissue area yielded the same reaction in 3–4 days. After primary sensitization, intervals of up to 8 week produced an accelerated secondary response. Significant differences between the reaction times of second set and third party allografts demonstrated recognition response specificity. This is the first report of an adaptive alloimmune response in gorgonians. A study by Frank & Rinkevich (2001) on the Red Sea hydrocoral *Millepora dichotoma* did not demonstrate a memory component. One of the genotypes usually overgrew the other in genetically determined directionality. In order to elucidate a possible memory component, first-set interactions were established in all possible pairwise combinations between three colonies, in eight replicates per combination. Interactions were followed for up to 8 week. Thereafter, interacting pairs were detached and either regrafted near the original contact area to form second-set assays or were challenged by third-party grafts. Additional delayed first-set assays were also established. Overgrowths of delayed first-set, second-set, and third-party grafts were followed again for 8 week. The mean overgrowth recorded in the second set of the interactions was indistinguishable from the first sets in all three colony-combinations.

During allospecific (as well as xenospecific) interactions, several effector mechanisms were further elucidated. Frank et al. (1995) revealed tissue growth without deposition of skeletal calcium carbonate in two Red Sea hermatypic cnidarians. Tissue contact between allogeneic colonies of the hydrocoral *Millepora dichotoma* resulted in unilateral tissue overgrowth. Skeletal material was not laid down for up to 10 week: tissue, which was loosely attached to the overgrown branch, advanced by up to 25 mm within the first 2 week. In xenogeneic interactions between the scleractinian coral *Cyphastrea chalcidicum* and the cirriped barnacle *Savignium dentatum*, tissues of the coral overlaid the plates of the barnacle without depositing calcium carbonate as long as the barnacles were alive (to 5 year). In both cases, the non-calcifying overgrowing tissue lacked polyps but appeared normal in histological section and contained typical cnidarian cells and endosymbiotic zooxanthellae. Jokiel & Bigger (1994), who examined regeneration as related to histocompatibility in the solitary coral *Fungia scutaria*, provided the first evidence of histocompatibility in a solitary coral. Discoid corolla of this free-living animal were cut with a rock saw and rejoined in various paired combinations and orientations of autogeneic (self-to-self), isogeneic (clone-mate to clone-mate), and allogeneic sections. Autogeneic or isogeneic sections of corolla with one section containing a mouth were joined along cut edges. In all cases, fusion of tissues occurred within weeks, followed by skeletal fusion within months. However, autogeneic or isogeneic sections rejoined along the uncut edges did not fuse. Isogeneic pairings between two sections with mouths did not produce either tissue/skeletal fusion or cytotoxicity at the interface.

Prospectus

The allorecognition/xenorecognition paradigm in reef corals, as in other invertebrates and vertebrates, is associated with the concept of self/non-

self recognition (Rinkevich, 1996a, b). Recognition of 'self' versus 'non-self' may represent two distinct ways that immune systems may be elicited by detecting the presence or absence of attributes that define self or by detecting the presence or absence of non-self attributes (Neigel, 1988). Despite the general acceptance of self/non-self recognition as a central immunological paradigm, effective immune responses exist that do not require self/non-self discrimination. For example, effector mechanisms that are directed specifically and very efficiently against phosphocolines and certain sugar transferases that are typically exposed on bacterial membranes are clearly not in the framework of self/non-self recognition assertion (Ohno, 1994). At this stage, we cannot answer simple questions about coral immunity such as 'what is self in historecognition?' and 'what is non-self?'.

Histocompatible experiments in corals also revealed a variable outcome with time, several studies documenting the existence of temporal reversals in xenogeneic (Chornesky, 1989) and allogeneic (Chadwick-Furman & Rinkevich, 1994; Frank & Rinkevich, 1994) encounters. These reversals were not due to other environmental factors; rather, they appear to result from inherent differences between interacting xenogeneic partners or allogeneic genotypes. However, it is not yet possible to determine whether the results were obtained from seasonal changes in allorecognition (as recorded in the mammalian immune systems; Nelson & Demas, 1996), from exhaustion (on a variety of ecological–physiological properties or cellular elements) of effector mechanisms (such as the reduction in number of phagocytic cells which are thought to carry cellular histocompatibility responses; Olano & Bigger, 2000), or from a genuine expression of late xeno–allospecific responses to a specific, chronic historecognition challenge.

A critical evaluation of historecognition in reef corals (criteria are summarized in Table 1), with an eye to the vertebrate immune responses, reveals that comparisons should not always be approached or judged by the mainstream immunological paradigms. However, when we examine the details of xenorecognition and especially of an allorecognition outcome (Table 1), almost any result found in the coral systems can be phenotypically matched with its counterpart outcome in the mammalian immune system. Hence, allorecognition systems in corals demonstrate all the variety of features that may be found in the complex structure of the vertebrate immune systems. In some aspects, such as in the appearance polymorphism of allorecognition responses (Rinkevich, 1996a, b) or the subtle expression of primary and late, secondary allospecific responses (Chadwick-Furman & Rinkevich, 1994; Frank & Rinkevich, 1994; Rinkevich et al., 1994; Table 1), the invertebrate systems even exceed levels found in vertebrates.

Studies of the last decade and former works have therefore clearly revealed that hermatypic corals, alcyonarians, and hydrocorals not only

Table 1. What alloresponses and xenoresponses in reef corals teach us? (summary of the cited literature)

The criterion tested	Allorecognition	Xenorecognition
Is historecognition precise and specific?	Yes	Yes
Is historecognition highly polymorphic?	Yes	Yes
Is historecognition capable of detecting fine difference?	Yes	Yes
Does historecognition express transitive/non-transitive relationship?	Yes	Yes
Does historecognition result in chimerism?	Yes	No
Does historecognition express an immunological memory?	Controversial	No
Immunological maturation?	Yes	No
Expression of delayed (secondary) responses?	Yes	Yes
Temporal reversals?	Yes	Yes
'Self' or 'non-self' recognition?	Controversial	Probably non-self

Yes = recorded in the literature, No = not found in controlled experiments.

represent a high variety of allorecognition operations on the morphological level, but also express delayed allogeneic responses such as cytotoxicity, overgrowth, reversal, and secondary responses that differ from a primary elicited outcome. The phenomenon of 'secondary responses' was evaluated in the hydrocoral *Millepora dichotoma* allogeneic responses (Frank & Rinkevich, 1994). These responses manifested the characteristics of selectivity and reproducibility, even when examined through time.

Histocompatibility responses in reef corals are shaped by both environment (Alino et al., 1992; Tanner, 1993; Genin et al., 1994; Aerts, 2000) and biological parameters (Salter-cid & Bigger, 1991; Alino et al., 1992; Rinkevich et al., 1993, 1994; Chadwick-Furman & Rinkevich, 1994; Frank & Rinkevich, 1994, 2001; Jokiel & Bigger, 1994; Frank et al., 1995, 1996, 1997; Bruno & Witman, 1996; Van Veghel et al., 1996; Griffith, 1997; Hidaka et al., 1997; Abelson & Loya 1999; Peach & Hoegh-Guldberg, 1999; Barki et al., 2002). Histocompatibility in corals, as in other organisms, relies on recognition elements (not yet disclosed in corals) as well as on effector mechanisms. Past studies have concentrated on revealing effector mechanisms as they were easily recognized and followed even in field studies. Recent studies are inclined to analyse and disclose recognition elements and genetics for historecognition, an approach most likely representing the early stage of a new era in the field of coral historecognition. Divergent allotypes versus convergent effector arms are the possible causes for the revealed repertoire of historecognition responses in corals where self/non-self specificity intermingles with aggression, feeding, and allorecognition.

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Epidermal structure of the scleractinian coral *Mycetophyllia ferox*: light-induced vesicles, copious mucocytes, and sporadic tentacles

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Key words: zooxanthellae, nematocysts, spirocysts, cnidocyst battery

Abstract

Three colony fragments of the scleractinian coral *Mycetophyllia ferox* Wells from Florida were observed in flow-through seawater aquaria under light and dark conditions. The colonies were then anesthetized and fixed for microscopic examination. Small vesicles formed across the epidermis in response to light as gastrodermis containing approximately 1.9×10^6 zooxanthellae cm^{-2} migrated into them. The vesicles flattened in the dark and the gastrodermis retreated to a clumped position. The epidermis is dominated by mucus cells with more than 6300 per mm^2. In contrast, there are very few epidermal cnidae. The polyps lack tentacles entirely, though small tentacles do occur, albeit sporadically, along the colline walls. Colline tentacles are expanded both day and night, and there is considerable intracolonial variability in the number of cnidae within them, ranging from as few as 316 to more than 3200 per mm^2 tentacle. There may be several small cnidocyst batteries containing both spirocysts and nematocysts (all microbasic p-mastigophores), but the principal battery is at the tentacle tip where cnidae are much more densely packed. There is considerable variation in the ratio of the two cnidae among tentacles in the same colony. Since the tentacles occur inconsistently and do not appear to expand, their functional role is unclear. Comparisons of epidermal characters are made with other members of the genus *Mycetophyllia*.

Introduction

Mycetophyllia ferox Wells (1973) is a scleractinian coral found throughout Florida, the Bahamas, and the Caribbean (Chiappone et al., 1996; Fenner, 1999), where it is often moderately common in 7–20 m depths (Lang, 1973). The Latin name 'ferox' likely refers to the ferocity with which this species attacks other corals with its nematocyst-laden mesenterial filaments (cf. Lang, 1973). However, little is known concerning the structure of either its mesenterial filaments or surface features. The status of *M. ferox* as a species has been unclear since Zlatarski (1982) synonomized it as a variant of *M. lamarckiana* on the basis of traditional skeletal characters. This study is part of a pilot project to assess soft tissue structure as one of several means of assessing coral species, and specifically sets out to characterize the epidermis of *M. ferox*.

Materials and methods

Three fragments of separate *Mycetophyllia ferox* colonies were collected according to permit restrictions at a depth of 16 m near Tennessee Reef light in the middle Florida Keys. The colony fragments (hereinafter referred to as fragments a, b, and c) were 180, 36, and 25 cm^2, respectively. Fragment 'a' was collected during August 2002. Fragments b and c were taken in February 2003. All corals were placed on running seawater tables

at the Florida Keys Marine Laboratory and allowed to recover for a week or more under a 12/12 light/dark cycle using fluorescent lighting. The colonies were then subjected to bright incandescent light for 12 h (400 μmol m^{-2} s^{-1}, 5600 K metal halide light source), then dark for another 12 h. This was repeated for an additional 24 h. The colonies were photographed under light and dark conditions, then anesthetized by adding concentrated $MgCl_2$ while they were enclosed in a 1-*l* container with seawater. Colonies were then fixed in cacodylate-buffered glutaraldehyde using methods described by Goldberg (2002a) and stored at 4 °C in 0.2 M cacodylate buffer until processed for light microscopy (LM), scanning electron microscopy (SEM), or transmission electron microscopy (TEM) using methods and instruments as referenced above. Material for LM and TEM was embedded in Spurr resin. Fixed tissues were usually excised from the skeleton using iridectomy scissors without decalcification. For larger SEM overviews, critical point drying produced large cracks across the specimen. In such cases the skeleton and overlying tissue were cut with a diamond saw, decalcified in 3% lactic acid/potassium lactate buffered to pH 4.0 overnight, then dehydrated and critical-point dried. For LM examination, fixed tissues were dissected into small (~1 mm) blocks and cover slipped to determine area prior to preparing squashes. Zooxanthellae from a total of 15 mm^2 of tissue were counted using an ocular micrometer that measured a 250 × 10 μm swath at 400×.

Individual tentacles were removed and cover slipped to determine area prior to preparing squash samples. When properly oriented, cover slipped tentacles flattened with the tip in the middle, making it possible to estimate the cnidae per mm^2 of tentacle epidermis. In a majority of cases the flat tentacle sample was less than a mm^2, but the number of cnidae was extrapolated to 1 mm^2 and counted as 1 out of the 15 mm^2 to be examined. The actual tentacular surface area examined was 6.46 mm^2 using the same magnification and micrometer area described above. Only mature cnidae with tubules visible in squash preparations were included in the totals. Mucocytes (=mucosecretory cells) were visible from the surface in fixed material and were counted from excised tissue squares using light microscopy without squash

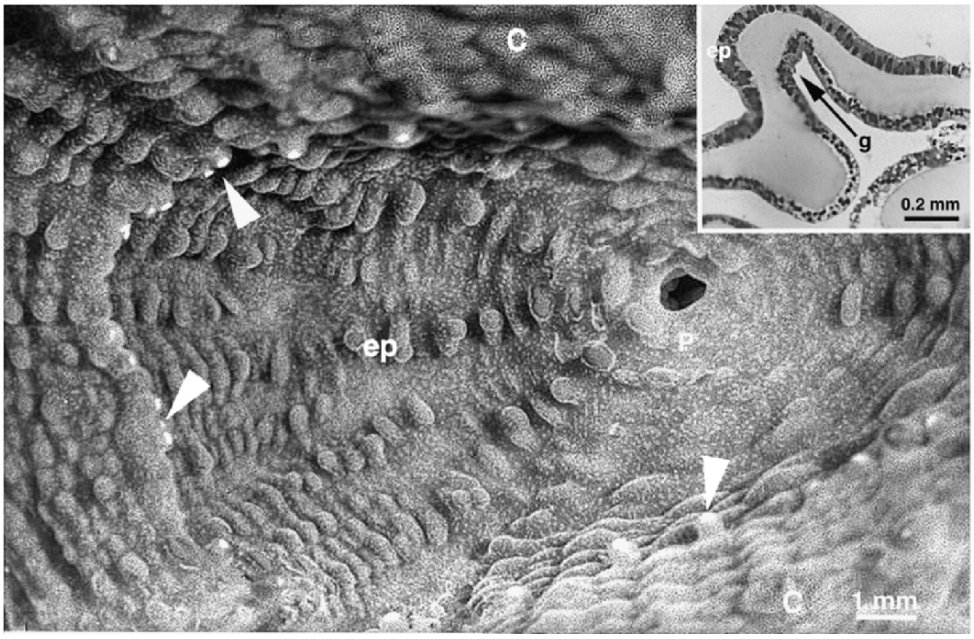

Figure 1. The epidermis of *M. ferox* (fragment b) exposed to light: note vesiculate epidermis (ep) allowing the gastrodermal zooxanthellae (g) a greater surface area for expansion (arrow, inset). A polyp (p) is surrounded by collines (c) with numerous small, laterally placed tentacles (arrowheads).

preparations. Fields of 1250 μm^2 instead of 2500 μm^2 were evaluated. Micrographs were prepared using Photoshop 5.5.

Results

Vesiculation of the epidermis

The epidermis of colonies in the light is clearly vesiculated (Fig. 1). Individual vesicles are polymorphic, ranging in area from 109 to 550 μm^2 of surface ($\bar{x} = 303 \pm 22\,\mu m^2$ SE, $n = 20$; height could not be accurately measured). The vesicles are lined with zooxanthellae and gastrodermis during the day. The epidermis becomes flattened and much less vesicular at night except for those immediately around the oral region of the polyps. In sectioned material the gastrodermis retreats into a flattened or clumped position, clearly reducing the surface area of zooxanthellae (Fig. 2). However, because of the small size of the vesicles it was impossible to reliably separate the underlying gastrodermis from either the flattened epidermis or the perioral vesicles that remain at night. Therefore the number of zooxanthellae cm^{-2} in the light-induced vesicles ($\bar{x} = 1.92 \times 10^6 \pm 45\,132$ SE; $n = 20$ 2500 μm^2 random fields in 15 mm^2 of epidermal vesiculae) cannot be reliably compared to nocturnal, non-vesicular tissue.

Epidermal cells

The outer epidermal surface is heavily ciliated (Fig. 3), but cilia have not been traced to individual cell types. The epidermis is thin, usually from 50 to 100 μm in the dark. The placement of epidermis, zooxanthellate gastrodermis, and the intervening mesogleal connective tissue is clearly shown by this figure. Following fixation of M. *ferox*, a green pigment layer on the surface of living tissue is removed, exposing the mucocytes (cell type verified by tissue sections) as epidermal white spots (Fig. 2). The undischarged mucosecretory inclusions are large (up to 38 μm long and 15 μm wide), spumous rather than electron-opaque, and stain a metachromatic pink with Toluidine blue, perhaps suggesting an acidic polysaccharide component. Mucocytes are clearly the dominant cell type in the epidermis (Figs 2 and 3) with a mean of 6315 mm^{-2} (± 583 SE; $n = 28$ 1250 μm^2 fields). The mucocytes explosively discharge their contents (Fig. 3 inset) in response to food (Goldberg, unpublished observations). In contrast to the mucocytes, epidermal cnidae are sparse. A 15 mm^2 area contains a total of 84

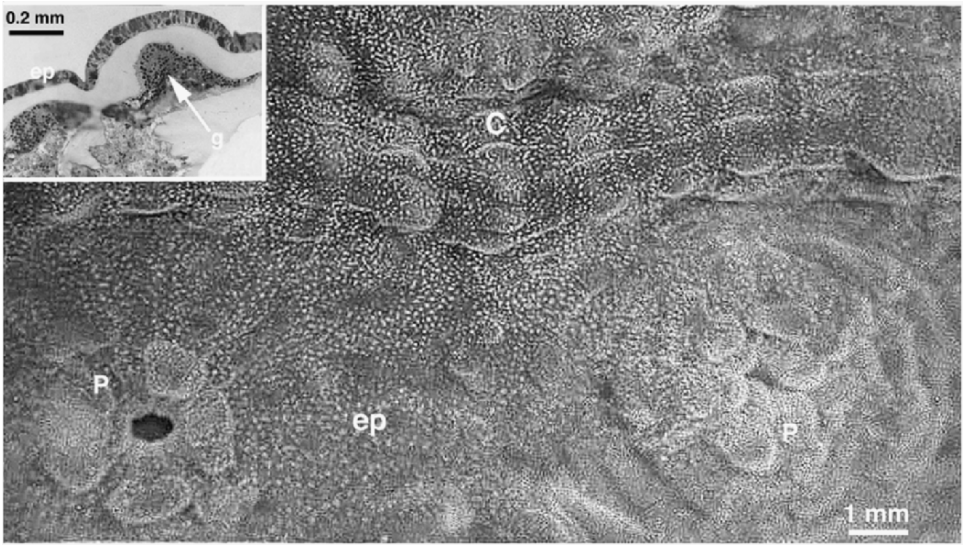

Figure 2. The epidermis between polyps flattens in the dark; the gastrodermis retreats to a clumped position (arrow, inset). Note the absence of colline tentacles in this sample (fragment a). None of the three tentacles in this 180 cm^2 fragment are shown. Each of the numerous white spots is a mucocyte. Symbols are as in Figure 1.

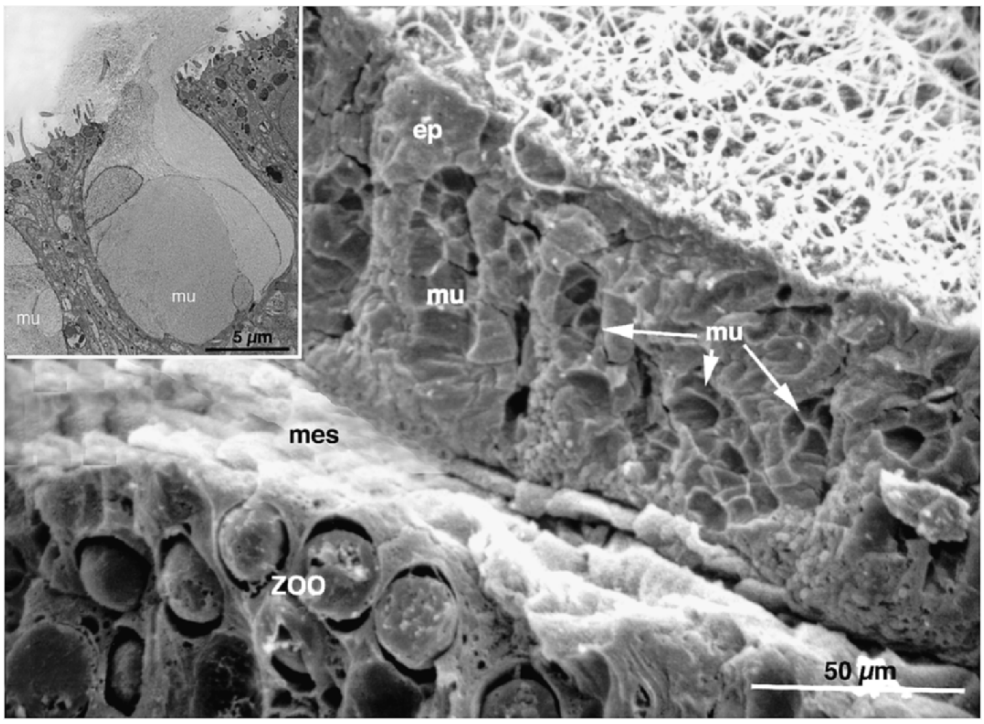

Figure 3. Ethanol cryofracture of *M. ferox* coenenchyme (fragment a, SEM): The epidermis (ep) is heavily ciliated and is dominated by spumous mucosecretory inclusions (mu). The zooxanthellae-containing gastrodermis (ZOO) is separated from it by mesogleal connective tissue (mes). Inset: The mucus is discharged explosively in response to food (TEM).

nematocysts and 24 spirocysts, fewer than 6 cnidae mm^{-2} (see below). Epidermal nematocysts are composed solely of microbasic-p mastigophores as indicated by Figure 4.

Tentacles occur inconsistently on the collines. Among the three specimens examined, the largest fragment ($a = 180$ cm^2) contained 3 tentacles (see Fig. 2) and fragment c contained 8. In contrast, tentacles occurred on almost every colline of fragment b and were particularly prominent around the edge of the colony (Fig. 1). Colline tentacles are small, typically 0.5 mm long and 0.7 at the base. Tentacle expansion was not observed in fed specimens in the laboratory, nor were they more prominent on specimens observed in the field at night (unpublished observations). However, all circumstances that might be associated with tentacle expansion have not been explored. Thus these observations must be regarded as tentative. The tentacles appear as shown in Figure 1 during both day and night. Zooxanthellae and gastrodermis extend close to the tentacle tips under lighted conditions and appear to recede within the tentacles to some extent in the dark (data not shown).

Most of the tentacle epidermis is composed of mucocytes (Fig. 4, large inset). However the tentacle tip is capped in white (Fig. 4, small inset) and contains a dense cluster of cnidae up to 225 μm in diameter, judging from cross sections of Spurr-embedded material. This apical 'cnidocyst battery' as defined herein is a wart or protrusion containing closely packed spirocysts as well as nematocysts. In the present case, the nematocysts are all microbasic-p mastigophores 65.6 μm $\pm$ 4.6 SD long and 5.9 μm $\pm$ 0.7 SD wide ($n = 10$). The spirocysts are 32.9 μm $\pm$ 3.6 SD long and 3.1 μm $\pm$ 0.5 SD wide ($n = 10$). Smaller cnidocyst batteries may occur elsewhere on the tentacle surface, however sub-apical batteries are not consistently present. Thus, most of the tentacle cnidae occur at the tip. Both types of cnidae are also found scattered throughout the epidermis, but they occur in batteries only within the tentacles.

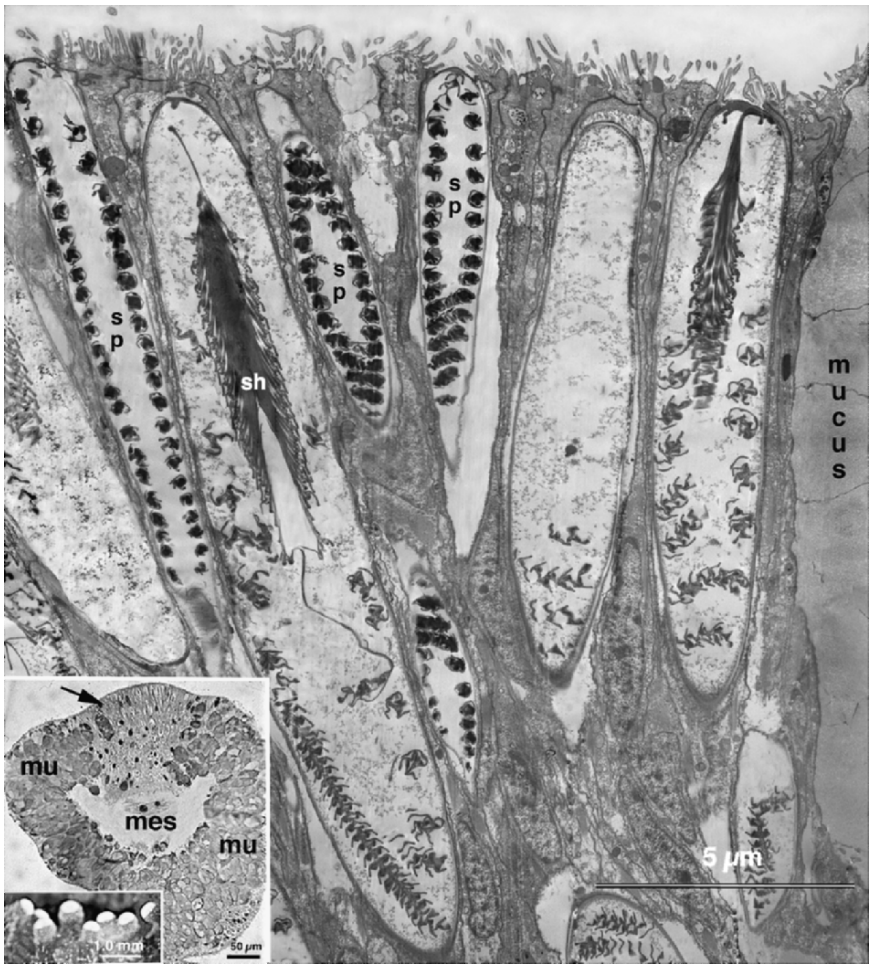

Figure 4. Colline tentacles and cnidae: Most of the cnidae are located within a cnidocyst battery (arrow, large inset) that is covered by a white cap at the tentacle tip (small inset). The epidermal cnidae are a combination of spirocysts (sp) and microbasic p-mastigophore nematocysts identified by the characteristic cleft in the shaft (sh) as it joins the tubule. This figure is a montage composed of six electron micrographs (TEM, fragment b).

The results from 12 tentacle squashes (all from colony fragment b) show considerable within-colony variation in both total cnidae and the proportion of spirocysts to nematocysts (Fig. 5). Although spirocysts are predominant in seven tentacles, the opposite is true in four others, and in tentacle 12 the ratio is 1.1:1. The number of cnidae within the tentacles ranges from 316 mm^{-2} (tentacle 1) to 3267 mm^{-2} (tentacle 11). The largest single tentacle area examined was 1.93 mm^2 and the smallest was 0.36 mm^2. A total of 9033 cnidae were counted, with a mean of 1322 mm^{-2} $\pm$ 260 SE. The density of cnidae within the cnidocyst battery is a different matter. There may be as many as 48 000 cnidae mm^{-2} ($\bar{x} = 33\,334 \pm 1579$ SE, $n = 20$ one-micron-thick sections from three tentacles). However, I estimate that the apical cnidocyst is no larger than 175×225 μm, $\sim$1/25 of a mm^2. Thus, the tentacle squashes represent a better estimate of cnidae density within tentacles, but sections are required to determine the density within individual batteries.

Discussion

Anthozoan 'pseudotentacles' are sometimes distinguished from feeding tentacles on the basis of

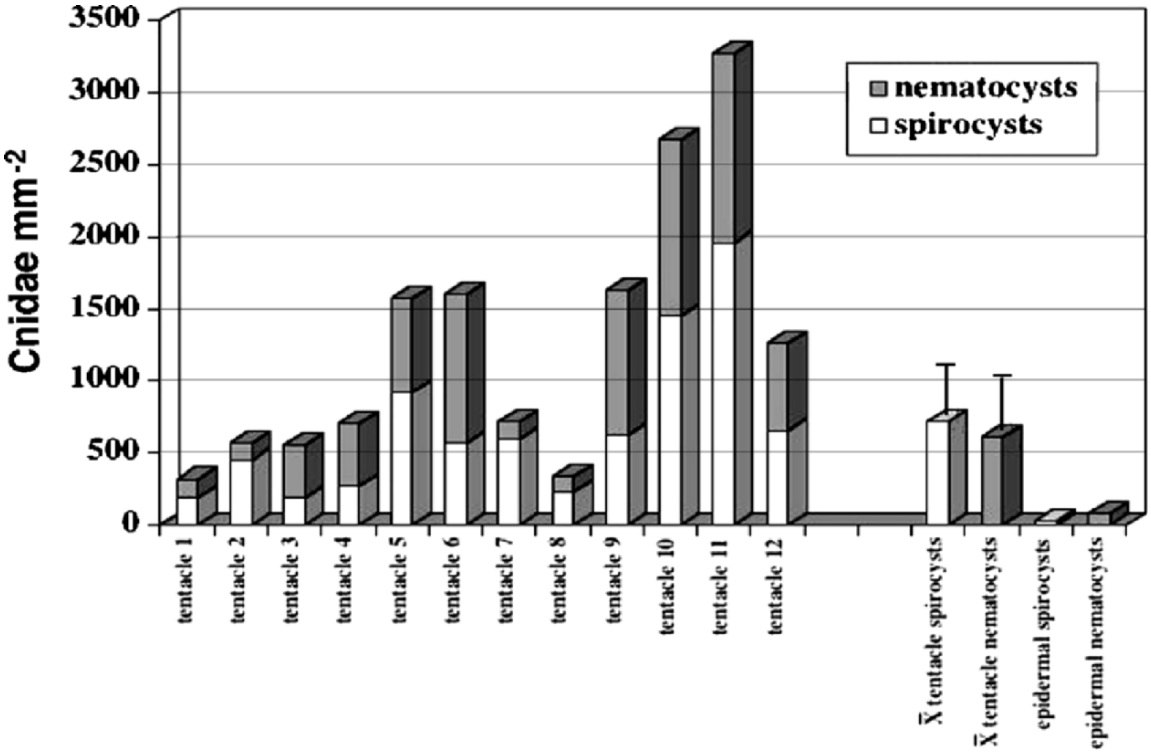

Figure 5. Histogram of cnidae mm^{-2} from 12 separate colline tentacle squashes (fragment b) showing within-colony variation: Note variable numbers of cnidae per tentacle and variable ratio of nematocysts to spirocysts. In general, spirocysts outnumber the nematocysts within individual tentacles (1, 2, 5, 7, 8, 10 and 11), although the reverse is true for tentacles 3, 4, 6 and 9. Bar cluster at right: The mean and standard deviation is shown separately for tentacular spirocysts and nematocysts. The non-tentacular epidermal cnidae are sparsely distributed and are shown as the total number found in 15 mm^2 of tissue.

morphology and activity patterns in response to light. The Caribbean anemone *Lebrunia coralligens* Wilson for example, possesses elongated, blunt-tipped tentacles that expand with increasing light intensity and are packed with zooxanthellae (Gladfelter, 1975; Lewis, 1984). Other more filiform or lanceolate tentacles expand in response to food. These 'feeding' or 'true' tentacles contain considerably fewer zooxanthellae, greater numbers of cnidae, and are expanded at night when prey is available (Sebens & DeReimer, 1977; Fricke & Vareschi, 1982; Lewis, 1984).

Most reef corals expand tentacles nocturnally although some are expanded continuously or only during the day (Abe, 1939; Porter, 1974; Lasker, 1979; Veron, 2000). However, a number of factors in addition to light may have an effect on tentacle expansion (Levy et al., 2001). While pseudotentacles as such have not yet been described from scleractinians, large epidermal vesicles extending up to 10 cm above the corallum occur in the Indo-west Pacific euphyllid coral *Plerogyra sinuosa* Dana. These grape-like protrusions form under lighted conditions and contain a zooxanthellae concentration of $1–2 \times 10^6$ cm^{-2} (Fricke & Vareschi, 1982; Vareschi & Fricke, 1986). The number of zooxanthellae in the smaller *Mycetophyllia ferox* vesicles lies within the higher end of this range (1.9×10^6 cm^{-2}), consistent with zooxanthellae density in other corals (e.g. Vareschi & Fricke, 1986, and contained references). This density also corresponds approximately to the average of $\sim 1.5 \times 10^6$ cells cm^{-2} expected in a monolayer of zooxanthellae (Drew, 1972), a figure that is considered as the baseline density for reef corals under normal environmental conditions (Muscatine et al., 1998, and contained references). Thus, while zooxanthellae density is unremarkable, vesicles appear to increase the surface area over which an approximate monolayer of symbionts can occur.

Other euphyllid Indo-west Pacific genera also form a vesiculate epidermis during the day (Veron, 2000), presumably providing a similar surface to volume advantage for their zooxanthellae.

Mucocytes are occasionally examined in cnidarians (see references in Goldberg, 2002a), but are rarely quantified. In *Mycetophyllia ferox* as well as in *M. reesi*, mucocytes are the most prominent epidermal cell type, and discharge is associated with feeding (Goldberg, unpublished; 2002a, respectively). However, there is a mean of 6315 mucocytes mm^{-2} in the species described herein, compared to 2906 in *M. reesi*. Correspondingly, there are more epidermal cnidae in *M. reesi* ($\bar{x} = 469\,mm^{-2}$) than in *M. ferox*, ($\bar{x} \leq 6\ mm^{-2}$), but neither number is particularly impressive, suggesting a greater dependence on mucosecretory mechanisms for sediment removal and food capture. Moreover, the overall scarcity of cnidae in the epidermis of both species directly contrasts with their aggressive mesenterial filaments (Lang, 1973) that may act as surrogate tentacles (Goldberg, 2002b).

The colline tentacles are an enigmatic feature of *Mycetophyllia* spp. All members of the genus appear to have colline tentacles (unpublished observations) with the exception of *M. reesi* which lacks both collines and tentacles (Goldberg, 2002a). The number of tentacles per colony in *M. ferox* is an inconsistent character ranging from 'numerous' to one per 60 cm^{-2} of colony surface. This curious feature should be verified by larger-scale sampling since it is based on a population of three colony fragments from one location. The virtual absence of tentacles in fragments a and c is also noteworthy in light of the inclusion of *M. ferox* in a group of corals that uses both mucus and tentacles during feeding (Lewis & Price, 1975). The occurrence of very small tentacles that appear not to expand is another curious feature that should be confirmed. However, it is consistent with these observations that the epidermal musculature is rudimentary and consists of only a few epitheliomuscular cells anchored in the mesoglea (unpublished TEM micrographs).

Cnidae in scleractinians are typically assessed by the proportional representation of different types, especially when comparing sweeper to normal tentacles (e.g. den Hartog, 1977; Hidaka & Yamazato, 1984; Hidaka et al., 1987). See Goldberg et al. (1990) for comparisons with other anthozoans. Cnidae density has been reported occasionally from anthozoan tentacles using squash preparations, however different authors employ different criteria. Schmidt (1982) counted cnidae per 100 epithelial cells, as did the earlier Hydra workers. Peach & Hoegh-Guldberg (1999) used cnidae per polyp milligram, whereas Thomason & Brown (1986) reported cnidae within tentacle and polyp squashes, but did not present them as a function of area. Fricke & Vareschi (1982) estimated the mean number of cnidae at 1600 mm^{-2} from sections of *Plerogyra sinuosa* tentacles in accord with the mean of 1322 found in this study. These are small numbers compared to the density of cnidae in the apical cnidocyst of *M. ferox*, and one can only speculate that tentacles with multiple cnidocyst or nematocyst batteries should have more than 1300–1600 cnidae mm^{-2}. When Harris (1990) examined sections of the corallimorpharian *Corynactis australis* (Edgar), he estimated that the acrosphere of each tentacle contained more than 500 000 nematocysts, whereas the more proximal portions contained an even higher number of spirocysts. Thomason & Brown (1986) reported that 12 scleractinian taxa vary widely in the number of cnidae per tentacle and may contain as few as 40 to as many as 15 000, depending on the species. It is apparent that more quantitative studies are needed to clearly define what the range of normality is for the density of scleractinian and other anthozoan tentacle cnidae.

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Adaptive bleaching: a general phenomenon

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Abstract

Laboratory and field data bearing on the adaptive bleaching hypothesis (ABH) are largely consistent with it; no data of which we are aware refute it. We generalize the ABH in light of these data and observations. The population of zooxanthellae within an organism is dynamic, the diversity of zooxanthellae is both surprising and difficult to ascertain, and field experiments demonstrate both turn-over in zooxanthella types and habitat-holobiont correlations. Dynamic change in symbiont communities, and the idea of an equilibrium or optimal community that matches the environment at a particular place and time, are concepts that underlie or emerge from much of the recent literature. The mechanism we proposed to explain responses to acute bleaching appears to operate continuously, thereby enabling the host-symbiont holobiont to track even subtle environmental changes and respond promptly to them. These findings enhance the potential importance of the ABH in the outcomes of acute bleaching, which can (1) accelerate this process of holobiont change, and (2) change the set of possible trajectories for how symbiont communities might recover.

Introduction

The adaptive bleaching hypothesis (ABH) may be concisely defined as the proposal that the loss of photosymbionts has the potential to allow some representatives of the host species to re-establish a symbiosis with a different dominant alga, resulting in a new holobiont (also referred to as ecospecies or host-symbiont unit) that is better suited to the altered environmental circumstances (Buddemeier & Fautin, 1993; Ware et al., 1996). Such a reshuffling has the potential to enhance long-term survival of the hosts, helping to reconcile the long-recognized paradox that coral reefs seem sensitive to environmental perturbation in the short term, but robust over geological time (Buddemeier & Smith, 1999). The framework of reefs is formed by scleractinian corals (and some other morphologically similar cnidarians) that, with few exceptions, are symbiotic with intracellular photosymbionts; the massive deposition of calcium carbonate by reef-forming corals is physiologically related, in ways that are still not fully understood, to possession of these symbionts (e.g. Al-Horani et al., 2003).

The hypothesized ABH mechanism rests on five fundamental assumptions: (1) multiple types of both zooxanthellae and host species commonly coexist; (2) a diversity of photosymbionts can live with many (but not necessarily all) hosts, and *vice-versa*; (3) different host-symbiont combinations may differ physiologically in aspects (particularly stress responses) that affect survival of holobiont, host, and perhaps symbiont; (4) bleaching provides the opportunity for repopulation of a host with different dominant photosymbiont (allochthonous or autochthonous); and (5) stress-sensitive holobionts have competitive advantages in

the absence of stress, which implies a reversion to stress-prone combinations under non-stressful conditions (Buddemeier & Fautin, 1993; Ware et al., 1996).

Knowledge of the symbiosis between the dinoflagellates commonly referred to as zooxanthellae and their animal hosts has grown enormously during the past decade. The host-symbiont system is now recognized as more dynamic than was previously thought. As Diekmann et al. (2002, p. 230) concluded, although 'the complexity and flexibility of the coral-zooxanthellae symbiosis is only beginning to be understood,' it is clear both that these 'symbioses are not evolutionarily constrained, species-specific associations … [and] that symbioses are not random.' According to LaJeunesse et al. (2003, p. 2047), the ABH 'assumes that symbiotic associations are highly flexible and adapt rapidly to environmental change.' We summarize the data that validate this perspective, reviewing them in terms of the five assumptions cited above, and generalize the ABH in light of these findings.

In the discussion that follows, as in the original formulation, we use 'adaptation' in the standard English (non-Darwinian) sense: '… modification of an organism or its parts in a way that makes it more fit for existence under the conditions of its environment…' (Merriam-Webster, 1997). Mechanistically, this may subsume any combination of what biologists define as acclimation, acclimatization, or adaptation. Although this usage has been the source of some confusion (see 'Challenges to the ABH' below) because it is at odds with the narrowly-defined, specialized use of the term in evolutionary biology, we consider communication with non-specialists to be of paramount importance.

As the pace of environmental change accelerates, the frequency of bleaching is rising. Enough of these natural experiments, in addition to some manipulative ones, have been conducted during the past decade to reject the simplest falsification of the ABH – the null hypothesis that there is no difference in symbiont composition of a host individual or colony before and after an episode of bleaching (e.g. Baker, 2001; Glynn et al., 2001; Toller et al., 2001a). More specific considerations are presented in the following sections.

Tests, validations, and uncertainties

Assumptions 1 and 2: diversity of hosts, photosymbionts, and holobiont combinations

Host diversity: The diversity of photosymbiont hosts has long been recognized. In addition to the nearly 800 species of reef-building scleractinian corals (Veron, 2000), zooxanthellae form symbiotic relationships with many taxa of anemones, octocorals, hydrozoans, foraminiferans, molluscs, and sponges. Although the diversity of hosts is well established, identification of symbiotic combinations is complicated by problems with identifying hosts (e.g. Santos et al., 2001) and misapplication of host names (e.g. *Parasicyonis* sp. of Rodriguez-Lanetty et al. (2002) is *Entacmaea quadricolor*, and *Rhodactis* (*Heteractis*) *lucida* of LaJeunesse (2002) is *Ragactis lucida*).

Photosymbiont diversity: When the ABH was initially formulated, the diversity of zooxanthellae was thought by many to be small, although the early idea that all zooxanthellae belong to a single species, *Symbiodinium microadriaticum*, had been abandoned (e.g. Trench & Blank, 1987, and summaries by e.g. Buddemeier & Fautin, 1993; Kinzie et al., 2001; Santos et al., 2002). Three clades, the units commonly used in taxonomic surveys of zooxanthellae, were recognized at the time the ABH was published; at present, seven clades, containing ~60 distinguishable types, are recognized (e.g. Santos et al., 2002; Baker, 2003, 2004). LaJeunesse (2002, p. 395) estimated that '40 or more distinctive types populate invertebrate hosts in the Caribbean' alone. These clades and types are coarse estimates of the true diversity of these organisms (e.g. Santos et al., 2001, and references therein; Savage et al., 2002a; Goulet & Coffroth, 2003a, b). Recognition of this diversity and accurate identification of individual taxa are critical if stability or change in associations is to be reliably assessed.

Holobiont (symbiosis) diversity: Coexistence of multiple types of zooxanthellae in a single host had not been convincingly demonstrated at the time the ABH was proposed, but was inferred by analogy with coexisting zooxanthellae and zoochlorellae in some sea anemones (e.g. Secord & Augustine, 2000) – a situation known in other symbiont systems as well (Anderson et al., 1998).

It is now well established that colonies of a single species of coral may contain multiple types of symbionts (Rowan et al., 1997; Santos et al., 2001, 2003), commonly one abundant and one or more others as a few individuals or in low densities (summarized by Douglas, 2003). LaJeunesse (2002) found a few symbiont types that occur in many taxa of hosts, but rare types that have high host specificity. On the basis of literature reviews and study of *Madracis* on Curaçao, Diekmann et al. (2002, p. 226) considered 'one zooxanthellae type is probably the norm.'

As it has become possible to detect finer distinctions among photosymbionts and smaller populations of them, more diversity has been recognized even in corals that had been considered specialists (e.g., Darius et al., 2000; Toller et al., 2001b). Diverse photosymbionts have been identified in cultures derived from a single host, but these may comprise only a subset of those living in the source animal in nature, in part due to the low probability of sampling types present in small proportions, in part because some types are differentially favored through selection imposed by culture conditions (Rowan, 1998; Santos et al., 2001).

Specificity: Specificity such as found by LaJeunesse (2002) is not inconsistent with the ABH, which postulates variability in host-symbiont specificity across the range of both potential partners such that some hosts and photosymbionts may establish symbioses with only one, or a very few, taxa.

Among taxa capable of multiple partnerships, the ABH postulates various behaviors: only some zooxanthella types may be taken up by a given host (perhaps due to recognition mechanisms between partners: e.g. Diekmann et al., 2002; Baker, 2003), only some newly-acquired zooxanthella types may survive, and, among them, fewer may persist or dominate the ultimate holobiont. Symbiont specificity through time on the scale of 10 years was convincingly shown in the field by Goulet & Coffroth (2003a, b) for the octocoral *Plexaura kuna*, but this specificity is a feature of one stage in the organism's life history. The primary polyp of a colony of *P. kuna* acquires its zooxanthellae from the environment and can take up those of more than one clade (Coffroth et al., 2001), but only those of clade B persist. Once established, the specific type of clade B zooxanthella associated with a genetically distinct host colony persisted through reciprocal transplants to different environments. Rodriguez-Lanetty et al. (2003) also showed stability in symbiont type at the clade level in sea anemones through time and space, but clade-level analysis does not reflect the functional diversity of possible photosymbionts.

Davy et al. (1997) followed the fate of allochthonous and autochthonous zooxanthellae through time in the sea anemone *Cereus pedunculatus*. Although zooxanthellae from the anemone *Anthopleura ballii* were taken up less readily than were those from other species, and autochthonous zooxanthellae were more persistent than allochthonous ones in the short term, in the longer term (36 weeks), densities of zooxanthellae from these two anemones were indistinguishable. Davy et al. (1997, p. 214) wrote 'The ability to establish symbioses with zooxanthellae from a range of sources may enable *C. pedunculatus* to adapt to different environmental regimes … Alternatively, it may simply increase the anemone's chances of survival should it lose all of its zooxanthellae and have to acquire new symbionts.' These are not mutually exclusive; together, they encapsulate the ABH.

Assumption 3: different host-symbiont combinations differ physiologically

Differences have long been recognized in environmental tolerances and preferences of the host taxa (e.g. Veron, 1995, 2000) and the algal symbionts, *in vivo* and *in vitro* (e.g. Ciereszko, 1991; Kinzie et al., 2001; Savage et al., 2002b). However, the relevant unit of analysis is the holobiont (e.g. Rowan, 1998). As Buddemeier & Fautin (1993) and Ware et al. (1996) stated, the 'ecospecies' is likely to have emergent properties not expected from knowledge of the partners in isolation (the additive model of their interaction by Ware et al. (1996) was a mathematical expedient). Toller et al. (2001b, p. 348) observed that for the animal partner, 'corals identified as members of the same species may not in fact be equivalent at the whole organism (holobiont) level,' and Rodriguez-Lanetty et al. (2001) demonstrated that one species of coral differs in photosymbionts with latitude.

Although different zooxanthella phylotypes differ physiologically, at least some photosynthetic traits do not correlate directly with characteristics of the phylotype (Savage et al., 2002b). Empirical observations of more than one zooxanthella type per colony, with different types predominating in different macrohabitats and microhabitats (e.g., Rowan et al., 1997), also make it clear that holobionts differ physiologically.

If holobiont physiology is an emergent property, rather than being additive or controlled by one of the partners, the long-running debate on which partner is responsible for observed tolerances (e.g. Edmunds, 1994; Diekmann et al., 2002; Bhagooli & Hidaka, 2003) is largely immaterial to the resulting pattern, however interesting and important it may be otherwise. Moreover, as stated by Burnett (2002, p. 105), although 'Ecophysiological plasticity of individual algal strains is limited … multiple strains may provide a degree of flexibility to host:symbiont associations.'

Assumption 4: bleaching provides opportunity for change of the dominant photosymbiont

Advances in understanding relevant to the ABH are not limited to those involving the identity of symbiotic partners or their interactions. Recognition of the variety, ubiquity, and range of intensities of bleaching processes points to the conclusion that catastrophic bleaching may merely represent the extreme end of a continuum that includes natural fluctuations in symbiont standing stocks over seasonal timescales, and catastrophic (or even visually detectable) bleaching may not be required for adaptive or acclimatory responses in ecospecies (Fitt et al., 2000, 2001).

The symbiont flora of an individual coral colony can undergo quantitative change (change in relative proportions of pre-existing algal taxa) and apparent qualitative change (appearance or dominance of a taxon not previously detected – e.g., Rowan et al., 1997; Baker, 2001; Toller et al., 2001b); the various combinations differ physiologically (e.g. Fitt & Warner, 1995; Warner et al., 1999; Toller, 2001b). Although there has not yet been unequivocal demonstration of qualitative change by allochthonous uptake as a prompt response to natural bleaching, uptake and persistence of allochthonous zooxanthellae have been demonstrated in field and laboratory experiments (Davy et al., 1997; Kinzie et al., 2001).

Hill & Wilcox (1998) and Toller et al. (2001a) found that severe bleaching allows establishment of novel associations, even if environmental conditions return to what they had been before bleaching. Moreover, zooxanthellae 'show patterns of dominance or prevalence similar to communities of free-living organisms' (LaJeunesse, 2002, p. 394). Thus, bleaching of any sort may open space in potential hosts for a wide variety of symbionts that are sorted not only in terms of their interactions with host and environment, but also among themselves (Baker, 2003, and references therein).

Changes in the dominant photosymbiont, whether from uptake of new partners following bleaching or from change in dominance of pre-existing internal populations, stem from stochastic processes, so the resultant holobionts may not be the same every time. Such change requires not only the ability to form diverse partnerships, but also, ultimately, the ability to acquire new (or multiple) symbionts from the environment, and the availability of such symbionts for acquisition.

Since some cnidarians do not acquire their symbionts maternally (= vertically) (e.g., Goulet & Coffroth, 2003b), infective zooxanthellae must generally be present in nature; Kinzie et al. (2001) demonstrated this and that uptake by bleached hosts is dose-dependent. Ralph et al. (2001) found zooxanthellae released from a coral host during bleaching to be physiologically functional; although not critical to assessing the ABH, this does mean that the population of possible partners is greater than if such cells were not viable.

Maternal transmission (see e.g., Benayahu & Schleyer, 1998, and references therein) of zooxanthellae is not necessarily identical with closed systems or highly specific symbioses. Diekmann et al. (2002) found no correlation of zooxanthella type with morphospecies of *Madracis*, in which zooxanthellae are transmitted maternally. Early embryos of the scyphozoan *Linuche unguiculata* acquire zooxanthellae maternally but later can acquire them from the environment – a system Montgomery & Kremer (1995) referred to as 'semi-closed.' Although the algae used in the experiments by Montgomery & Kremer (1995) were all autochthonous, the results indicate that maternal

acquisition may not preclude acquisition from the environment as well. Thus, in this respect also, the system may be more flexible than had been thought; rather than the vertical and horizontal (or closed and open) transmission alternatives that Rowan (1998, p. 412) thought might imply two 'evolutionary scenarios, probably with different consequences for symbionts diversification,' the two may be the end members of a continuum.

Assumption 5: stress-sensitive holobionts have competitive advantages in the absence of stress

Recent data are consistent with the model of the ABH by Ware et al. (1996) postulating that successive bleaching events eliminate types of photosymbionts sensitive to a particular stress, while types more resistant to the stress remain: Glynn et al. (2001) showed that zooxanthellae of Clade D have spread in the eastern Pacific recently at the expense of those of Clade C, and Toller et al. (2001b) inferred that zooxanthellae of clade D (see taxonomic assignments of Baker, 2003) dominate in certain circumstances not by dint of direct competition between algae, but because they tolerate conditions others do not.

In the experiments of Kinzie & Chee (1979) and Fitt (1985), autochthonous photosymbionts ultimately persisted whereas allochthonous ones did not. Reversion to the original combination is what might be expected under the ABH since the experiments were being conducted under conditions known to be favorable to the original holobiont – provided that there is some inherent preference for the stress-intolerant taxa, and/or that they contribute to superior holobiont fitness under low-stress conditions. This postulate of the model of Ware et al. (1996) is supported by general observations (only stress-resistant types should remain after repeated bleaching episodes without some countervailing advantage of the less resistant types), and by recent data of Van Oppen & Berkelmans (R. Berkelmans, personal communication). However, this is clearly one of the areas most urgently in need of additional research.

The issue of competitive advantage focuses on why the ABH is difficult to test by single experiments – it deals with population-level features, with responses that occur stochastically, and with the global environment over a long time, in all of which rare events may be very important and in which correlative data are essential. These issues are dealt with more extensively by Buddemeier et al. (2004).

Challenges to the ABH

Virtually all evidence adduced thus far is consistent with, much supportive of, and none fatal to, the ABH, contrary to the assertion of Hughes et al. (2003, p. 930) that 'A hypothesis that bleaching is 'adaptive,' increasing coral fitness by facilitating expulsion of susceptible zooxanthellae species and uptake of more resistant ones, has not been supported by observations on the fate of bleached corals.'

Some arguments purporting to falsify the ABH are based on single experiments or limited observations that are not robust tests of an hypothesis that operates stochastically and that deals with long times and broad patterns. For example, occurrence of an apparently single type of photosymbiont in a host organism or species, the same type of photosymbiont before and after bleaching, or the absence of a simple correlation between habitat and symbiont type, does not invalidate the ABH. In the case of highly specific symbioses, no change would be expected (or possible). However, for non-specific symbioses, the ABH posits a stochastic process, not a deterministic one, so bleaching need not inevitably lead to a changed symbiont flora (Buddemeier et al., 2004), and the probability of change depends on the variety of photosymbionts in the environment or the host – the greater the variety, all other things being equal, the more likely change will occur.

A practical issue affecting the validity of some purported tests is the coarseness with which diversity within the currently recognized clades can be resolved. Failure to observe replacement of one member of a clade by another may reflect absence of change or simply the inability of the analytical techniques used to detect the change that occurred; the same is true for detection of multiple symbionts. For example, Kinzie et al. (2001) observed that various subpopulations of zooxanthellae that were indistinguishable by the methods used in that study had very different physiological responses to changes in temperature, but Santos et al. (2002),

applying different molecular techniques to the same samples, found that unique responses corresponded to unique genetic sequences.

Misunderstanding or misstatement of the ABH has been persistent. Hoegh-Guldberg (1999) confined his critique of the ABH to heat stress, and Hoegh-Guldberg et al. (2002, p. 602) stated 'We consider that the evidence in favour of the ABH remains scant in the absence of observations that the genotypes of symbionts in corals become more thermally robust during and after mass bleaching' and 'we cannot conclude that bleaching favours new host-symbiont combinations that guard populations of corals against rising sea temperature.' Bleaching need not be 'mass' in nature, and the ABH is not restricted to heat stress. Ware et al. (1996, p. 201) used temperature as 'a convenient mathematical representation of a synergistic stress'; in fact, Fitt et al. (2001) posited synergism as well as dose-dependence among stresses. Further, Hoegh-Guldberg et al. (2002, p. 602) criticized a study by Baker (2001) by asserting that Baker 'is unable to distinguish newly invading genotypes from other rare genotypes that are already present in the host and which simply increase in proportion after conditions change.' As Baker (2002) pointed out in his rejoinder, this is irrelevant as far as the ABH is concerned.

The statement by Reaser et al. (2000, p. 1503) '… perhaps [corals] and their zooxanthellae may become more stress-tolerant (Buddemeier & Fautin, 1993; Ware et al., 1996)' implies they have misinterpreted the ABH as positing the evolution of stress-tolerance in the individual partners, as opposed to formation of more tolerant holobionts from individual partners that evolve new characteristics slowly.

The ABH has also been misstated in teleological terms. Hill & Wilcox (1998), for example, wrote 'corals bleach as the environment changes *in order to* acquire a new algal complement which is better adapted to current conditions' (p. 281, italics ours) and 'host invertebrates will expel and replace their resident algae with a symbiont more adapted to current environmental conditions' (p. 287). Variant teleological misstatements include 'there is no evidence that corals bleach specifically to exchange one genotype of zooxanthellae in their tissues for another' (Hoegh-Guldberg, 1999, p. 856) and 'The ABH assumes that bleached corals favour new host-symbiont associations that optimize survival' (Hoegh-Guldberg et al., 2002, p. 602). The ABH *does not* state that a coral (or any animal host) *chooses* its symbionts; it *does* state that bleaching provides an opportunity for new combinations to arise, among which selection may occur for well-adapted ones (i.e., those that optimize survival), but does not *require* the combination to change.

In fact, although Hill & Wilcox (1998) interpreted their results as not supporting the ABH, the results are entirely consistent with it. They argued the pre-existing type of zooxanthella should have reinfected the sponge after bleaching since it represents the best-suited combination, but that a new type did. Alternative interpretations of their results are not mutually exclusive: one interpretation is that the new combination was due to the types of zooxanthellae available in the environment, another is that the new combination was actually superior.

Another source of debate about the ABH centers on the definitions of adaptation *vis à vis* acclimation and acclimatization (see Gates & Edmunds, 1999). The phenomenon, one aspect of which is loosely described as 'bleaching,' is an adaptation that we propose has allowed scleractinians to switch partners which, in turn, has allowed them to survive the large climate shifts through which they have persisted. As Baker (2002, p. 602) stated, 'if more rapid symbiont change proves beneficial, then bleaching has adaptive value.'

If the host-symbiont combination is regarded as a physiological unit, substituting a different zooxanthella type or changing the proportion of types alters the attributes of that unit. This is not acclimation in the usual biological sense since the genetic make-up of the unit has been altered, but contrary to the specialized biological use of the term adaptation, this does not require evolutionary time – it can be essentially instantaneous. Misinterpreting our use of the term 'adaptive,' Hoegh-Guldberg et al. (2002, p. 602) asserted 'The ABH … necessitat[es] rapid evolutionary adaptation (that is, genetic change) by populations of reef-building corals and their symbionts.' The semantics of this debate are discussed by Baker (2004) and Buddemeier et al. (2004).

Discussion and conclusions

We interpret recent evidence as supportive of our perspective that the animal-zooxanthellae relationship can respond dynamically to the environment, both biotic and abiotic. By analogy, lichens, a similar system consisting of algal and fungal partners, is not the tightly co-evolved symbiosis it had been considered (e.g., Piercey-Normore & DePriest, 2001). In fact, we conclude that our initial emphasis on adaptive response to obvious bleaching is too restrictive. The mass bleachings that appear to be increasing in severity and frequency are the results at one end of a continuum of environmental change over space and time. The zooxanthella density of an animal host varies through the year (Fagoonee et al., 1999; Fitt et al., 2000, 2001), blurring the definition of bleaching and contradicting the dogma that the population of zooxanthellae is kept within narrow limits by the host (e.g., Falkowski et al., 1993). Therefore, there seems to be a virtually continual opportunity for creation of new holobionts upon which selection can act and thus provide prompt, dynamic response of the symbioses to changing environmental conditions. In a neutral population genetic model for the cnidarian-algal symbiosis that she developed, Orive (submitted for publication) calculated that about one algal cell taken up from the environment every 50 days is sufficient to balance loss of genetic diversity due to drift, bottle-neck effects, etc. and thereby maintain within-organism zooxanthella diversity at its local maximum.

The existence and operation of the mechanisms proposed as part of the original ABH have been confirmed in many cases, and conclusively refuted in none. It now appears that adaptive change in the dominant symbiont can occur over the entire range from normal background fluctuations in algal populations to acute bleaching events. In the latter, the stress may (1) accelerate the process of change, and/or (2) change the set of possible trajectories for how these communities might recover by dramatically changing the starting abundance of each symbiont type and thereby shifting the system out of a region of local stability (where symbiont community structure tends to return to the same equilibrium after minor disturbance) into a region of relative instability (where many more recovery outcomes are possible).

Although further research is needed to clarify the environmental and evolutionary importance, detailed mechanisms, and extent of the ABH, we cannot improve on the statement by Baker (2002, p. 602): 'The real question is not whether coral-algal associations can adapt by recombining, but rather how, and over what timescales, they do so.'

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Observations of a new source of coral mortality along the Kenyan coast

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Abstract

In early 2002 coral mortality occurred along 600 km of coastline from Tanzania to Kenya. *Astreopora, Echinopora*, and *Montipora* species were severely affected, with *Montipora* being nearly eliminated from Kenyan reefs. *Acropora*, *Platygyra*, *Goniopora*, and massive *Porites* were also affected; however, *Porites* and *Goniopora* rarely died and often recovered, whereas death for most other species occurred within 2 weeks. In *Echinopora* and *Montipora*, a dull ashy tissue color and brittle skeletons characterized the early stages of this event with a mucus layer on the tissue surface in intermediate stages. Mucus and embedded debris then disappeared and surfaces were left covered in a white calcareous dust that sometimes capped a black layer. *Astreopora* tissues became dull and pale, and seldom produced mucus; eventually the skeleton became bare and white. Either a colorless translucent or brownish thin margin of tissue was visible between living tissue and bare skeleton, depending on species. Scanning electron micrographs of affected corals revealed the presence of fungi. Histology and staining showed that the fungi were mostly in the three genera that died from the syndrome and it may be that fungi invaded and killed corals weakened by another unidentified pathogen.

Introduction

Coral diseases or syndromes are increasingly being recognized as a major cause of coral mortality (Peters, 1997; Richardson, 1998; Harvell et al., 1999; Porter, 2001). Most investigations are from the Caribbean where diseases have caused large-scale mortalities of corals (Aronson & Precht, 2001; Aronson et al., 2002). Few investigations have been conducted into coral pathologies in the western Indian Ocean, and the extent to which diseases kill coral and change reef ecology there are not known. Many of the common 'band' diseases have been identified in that region (Antonius, 1988a, b; Korrubel, 2000; Ravindran et al., 2001; Ben-Haim & Rosenberg, 2002; Riegl, 2002); however, the causes and ecology of these diseases are poorly understood. We summarize investigation into a mass coral mortality that occurred along a 600-km stretch of the East African coastline in 2002. We describe the changes occurring in the corals as they became morbid and often died, and investigate potential causes and consequences to the coral community.

Materials and methods

This study of coral mortality combined the results of visual observations in the field, histological and scanning electron microscope (SEM) observations, benthic line-intercept transects, and water temperature measurement. It was instigated as part of an annual monitoring program undertaken since 1991 in seven Kenyan reef sites during the warm northeast monsoon between December and March. Each site was visited for a minimum of four consecutive days so at least 28 field days were accumulated during the sampling period.

Water temperature measurements began in 1997 with *in situ* gauges (Hobo Temp-Onset Co.) on the reef. Gauges were placed in the same position each year in the Mombasa Marine National Park (MNP) under a coral head at 1 m depth at low tide (Kenya has a 4-m tidal range), hidden from view and sunlight, cemented to the substratum, and programmed to record a measurement every 3 h. For the purposes of this study, we compared temperature data from 1 July 2001 until mid-February 2002, the period before mortality was observed in 2002, with data from two periods: 1997–1998, when bleaching was observed (McClanahan et al., 2001) and 2002–2003, when no bleaching or coral mortality was observed. Differences between years were tested with a single-factor ANOVA.

In 2002, visual observations were made between January and March in eight reef locations along ~200 km of coastline of southern Kenya and were also based on discussions with other field investigators (J. Church and E. Vereij), which extended the cumulative observations to ~600 km of coastline. Observations were reported to the United States National Oceanic and Atmospheric Administration (NOAA) coral-list server in an attempt to determine the extent of the mortality, but no observations outside of East Africa were reported.

Tissues for histology and SEM were collected with a 20-mm corer in March 2002 from the Mombasa MNP. Samples were collected in pairs, one at the margin between dying and living coral tissue, and one 'control' sample from healthy tissue on the same colony. Specimens were fixed in seawater formalin for 24 h, and stored in 70% ethanol. For SEM, tissues were dehydrated in an acetone series, critically point-dried, and sputter-coated with gold prior to observation in a Jeol 5600LV variable pressure SEM at up to 1000×. Histology samples were decalcified in EDTA (10% w/v, adjusted to pH 7.0 with NaOH); the solution was changed twice daily. Tissues were dehydrated through a graded series of ethyl alcohol, cleared in xylene, infiltrated with paraffin, and embedded following standard protocols (Howard & Smith, 1983). Sections were cut at 4 μm and stained with

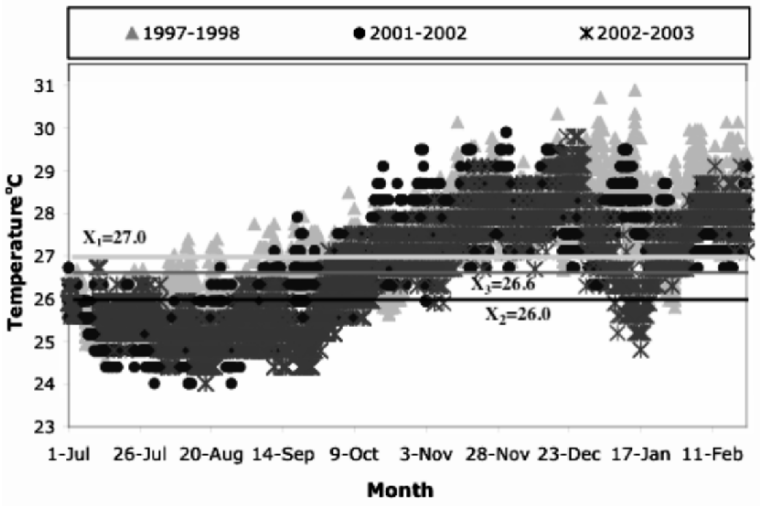

Figure 1. Water temperature in 2001–2002 compared to years in which coral bleaching was observed (1997–1998) and no bleaching or mortality were observed (2002–2003). Data from *in situ* gauges in the Mombasa Marine National Park collected every 3 h at 1 m.

Mayer's hematoxylin and eosin, alcian blue/periodic acid-Schiff reagent (PAS), Twort's Gram stain, and Grocott's methenamine silver (GMS) (Howard & Smith, 1983).

Scleractinian coral diversity and cover were estimated at seven sites along 12–18 10-m benthic line-intercept transects per year. Hard corals were identified to genus, and the length of each genus under the 10-m line was measured to the nearest centimeter. The relative abundance of each coral genus for all sites combined was estimated by dividing the cover of each genus by the total coral cover. We plotted relative abundance of these genera over time and coral cover for all and affected corals from 1995 to 2003.

Results

Water temperatures

In 2001 and 2002 prior to the coral mortality event, temperatures were 26.6 ± 1.3 (SD) °C and never exceeded 30 °C (Fig. 1), which was significantly lower (ANOVA, $F = 74.1$, $p < 0.0001$) than in the coral bleaching year (1997–1998), when the mean temperature was 27.0 ± 1.1 °C. Conversely, temperatures during 2001–2002 were higher than during 2002–2003, when no bleaching or diseases were observed, and the mean was 26.0 ± 1.1 °C. The change in the monsoon winds in January is often associated with cooling of the water; this is clearly seen in 2002–2003 but was less evident in 1997–1998 and 2001–2002 periods when bleaching and the coral mortality were observed, respectively.

Field observations

Observations of coral mortality were made between January and February 2002, when water temperature was 28 °C. The gross signs of morbidity and mortality were significantly different from bleaching or other local sources of coral mortality observed previously (Fig. 2). Only three

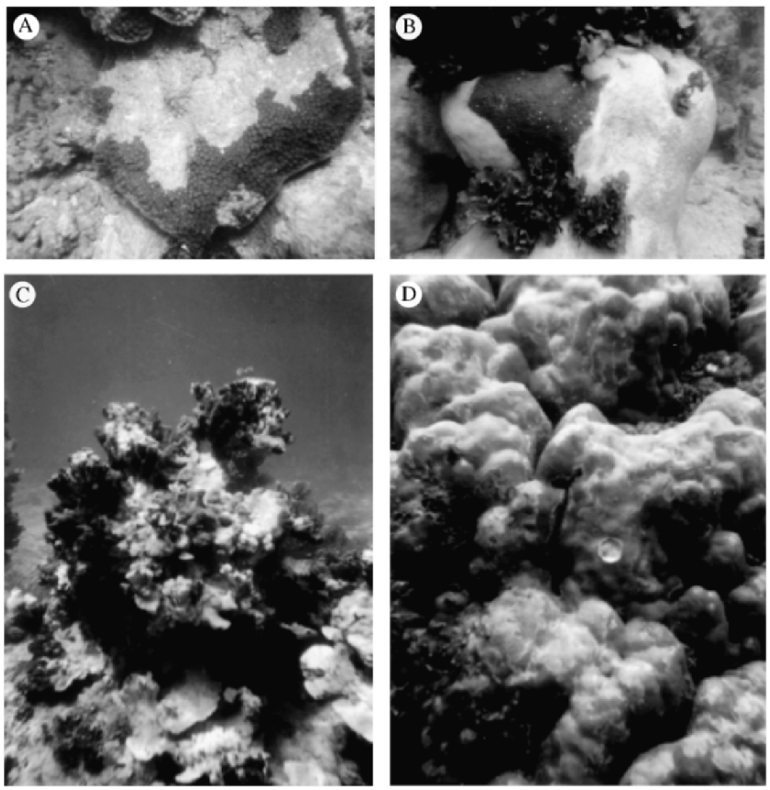

Figure 2. Photographs of affected corals in the field in February 2002. (A) *Echinopora gemmacea*, (B) *Astreopora myriophthalma*, (C) *Montipora aequituberculata*, and (D) *Porites lutea* – with an area where a core was removed for microscopic analyses.

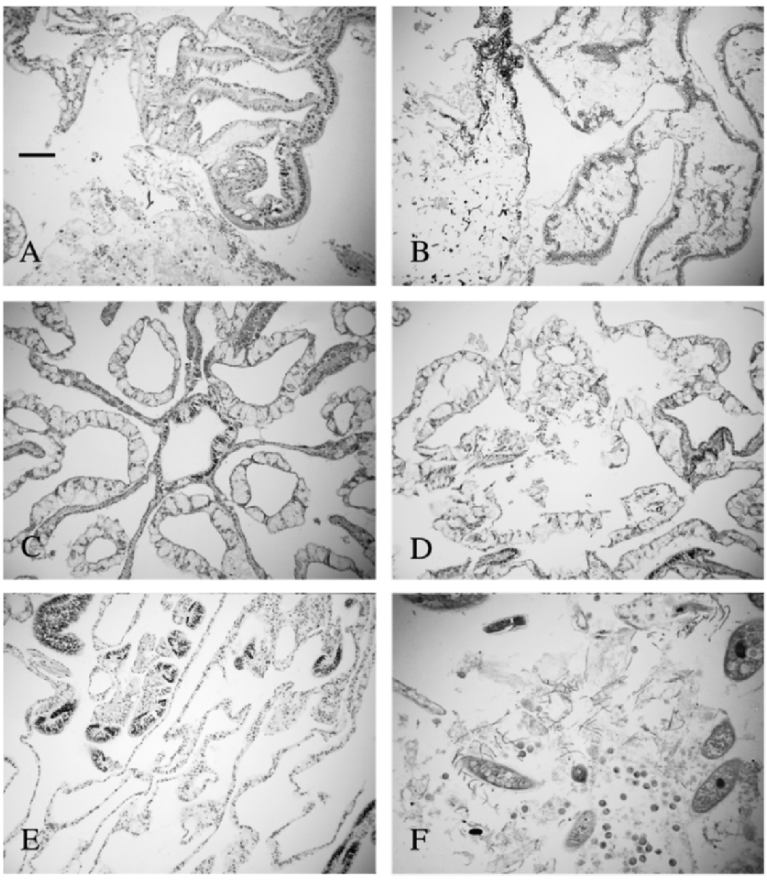

Figure 3. Light micrographs (200×) of coral tissues stained with Mayer's hematoxylin and eosin. (A) Necrosis and lysis of cells adjacent to intact tissues of *Astreopora myriophthalma*, (B) fungal mat along surface of *Echinopora gemmacea*, (C) intact, (D) lysed polyps of *Porites lutea*, (E) intact tissues of *Montipora aequituberculata* adjacent to lesions containing necrotic tissue, and (F) lysed tissues containing cellular debris, ciliates, bacteria, and fungi. Scale bar 37 μm.

of 24 genera of corals present at these sites suffered mortality; four other genera showed similar signs of disease, but recovered. In the early stages of the death of *Montipora aequituberculata* and *Echinopora gemmacea*, the affected corals developed an ashy dull coloration with a brittle or weak skeleton, while in the intermediate stages they became covered with mucus that collected debris. Once the mucus and debris cleared, a white calcareous dust was left on the surface and sometimes a black color was found underneath this dust. Death was very fast, often taking less than 14 days, and large monospecific stands of *M. aequituberculata* 20×20 m^2 were killed in less than a month. Effects on *E. gemmacea* were more variable with patches of bare skeleton within areas of apparently normal tissue. *Astreopora myriophthalma* developed a dull pale color, seldom produced mucus, and then left a bare white skeleton. *Porites lutea* turned from brown to ashy gray and then became covered with mucus, but mortality was rare. The syndrome disappeared in April and was not seen afterward.

Tissue observations

Astreopora myriophthalma

Extensive necrosis and lysis were observed in lesions of affected samples (Fig. 3A). A biofilm of bacteria was present on the outer epidermis. Gastrodermis contained pyknotic cells. *Symbiodinium* (zooxanthellae) lacked normal architecture. Rare filaments remaining where the skeleton was removed in the sections were PAS-negative and fungal-like filaments were GMS-negative. The

apparently healthy sample from the same colony also had surface bacteria and foci of necrosis, indicating that this tissue also had been affected and might soon disappear from the skeleton. Ova were beginning to develop in the apparently healthy sample.

Echinopora gemmacea

The apparently healthy samples were developing ova and spermaries. Both apparently healthy and affected tissues had foci containing cells that were necrotic with pyknotic nuclei, and other foci of complete cell lysis. A sharp delineation between lysed and healthy tissue was apparent. Many bacteria were observed along the epithelial surface. GMS-positive fungi were found in both healthy and affected coral specimens, sometimes forming a dense mat with a distinct margin on the coral surface (Fig. 3B). Fungal filaments were also present in the spaces where skeleton was removed between polyps.

Porites lutea

The apparently healthy tissue samples were normal in cell and tissue architecture and staining reactions; spermaries were developing. A mucus layer containing bacteria was apparent along the surface of tissues in the affected samples. Examination of tissues suggested the cellular changes began at the oral surface and penetrated inward. Tissue within the gastrovascular canals did not appear to be adversely affected. Foci of tissue necrosis and lysis were observed less frequently than in the other genera examined (Fig. 3C and D). Fewer bacteria were observed and GMS-positive fungal filaments were rare.

Montipora aequituberculata

The apparently healthy samples displayed normal tissue architecture and staining (Fig. 3E). A mucus layer was present on the oral surface tissue in the affected samples. Large foci of necrotic tissue

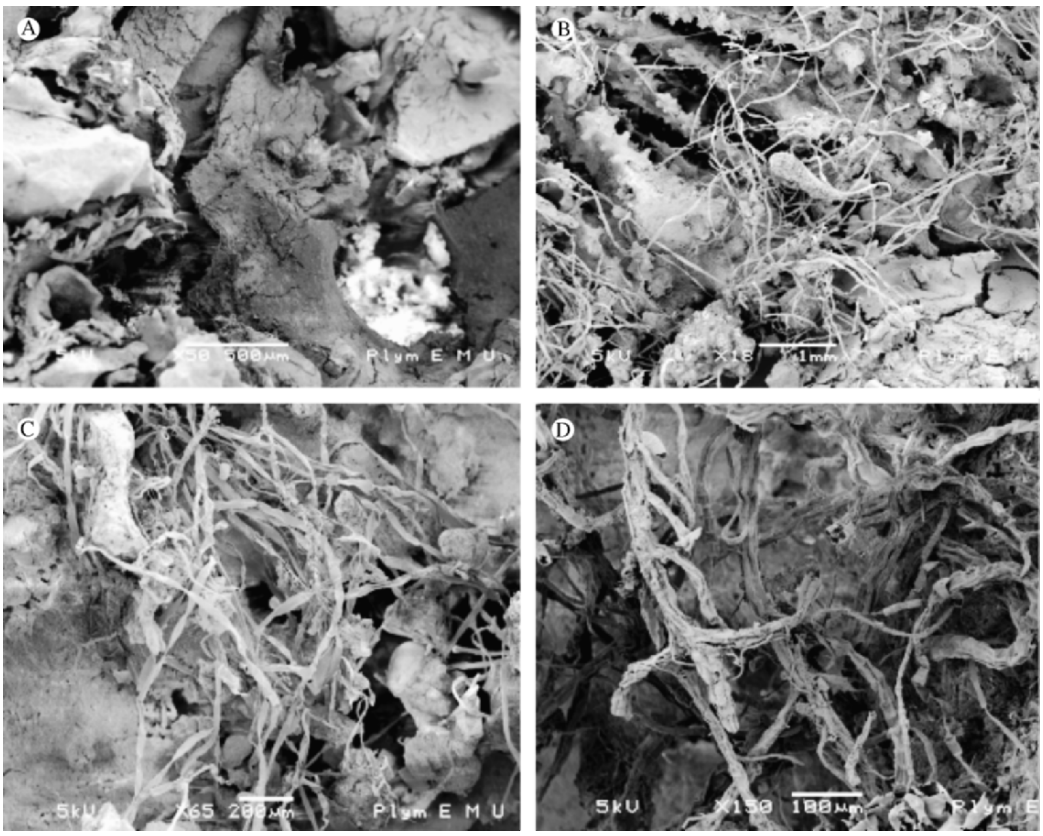

Figure 4. Scanning electron micrographs of (A) control (apparently healthy) and affected (B–D) coral tissue at three levels of magnification.

contained cell debris, copepods, bacteria, a few filaments of PAS-positive fungi, and ciliates, which appeared to have engulfed released zooxanthellae (Fig. 3F). The epidermis near lesions was sloughing or not present, and the gastrodermis contained pyknotic nuclei. More acidophilic granular cells in the diseased compared to the apparently healthy samples suggested lysosomal activity was increased in the diseased samples. Swelling and lysing of tissue cells appeared to occur before bacteria were present. Mesenterial filaments showed increased vacuolation. Fungal filaments were observed and some samples were PAS- and GMS-positive.

Goniopora stokesi

Both the apparently healthy and diseased samples showed foci of necrosis; ova were developing in both. Sloughing of the epidermal layer occurred. Calicoblastic epidermis was more columnar than normal and showed an increase in eosinophilic granules. GMS-positive filaments in the skeleton were rarely observed. Tentacles were missing from polyps in the diseased samples.

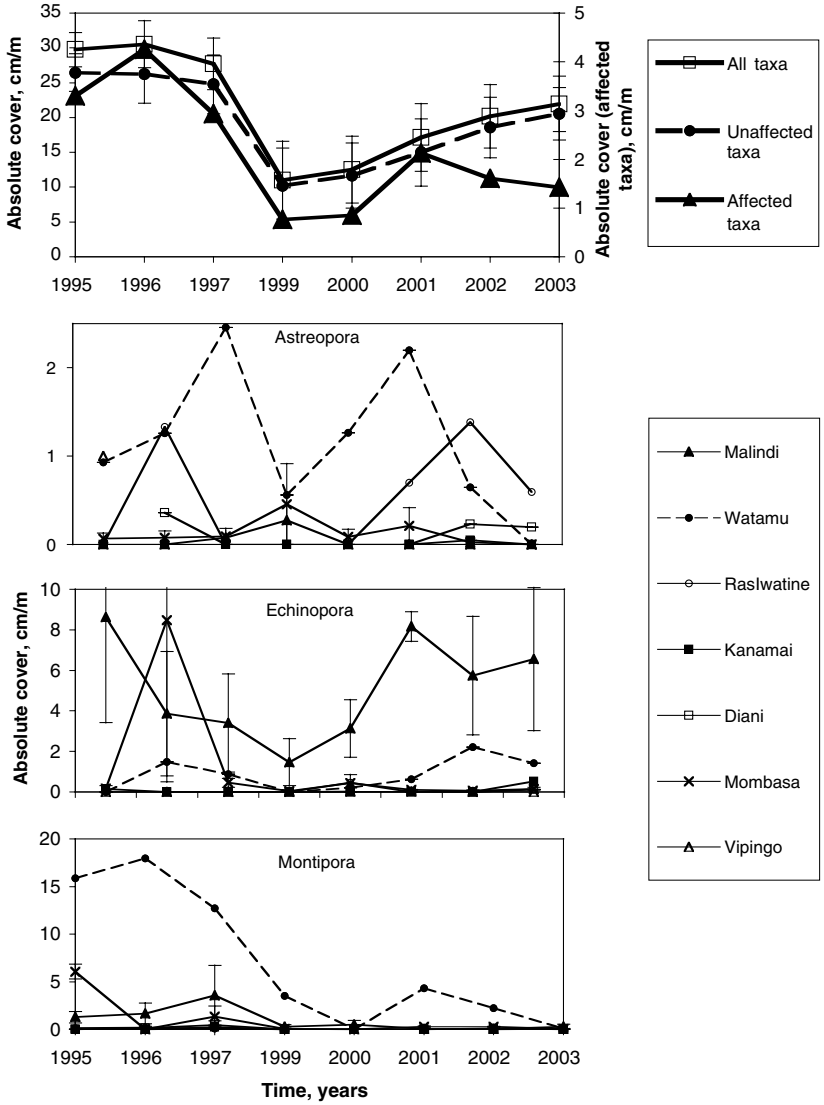

Figure 5. Changes in total coral cover and pooling species most affected by the mortality and each of the most affected taxa.

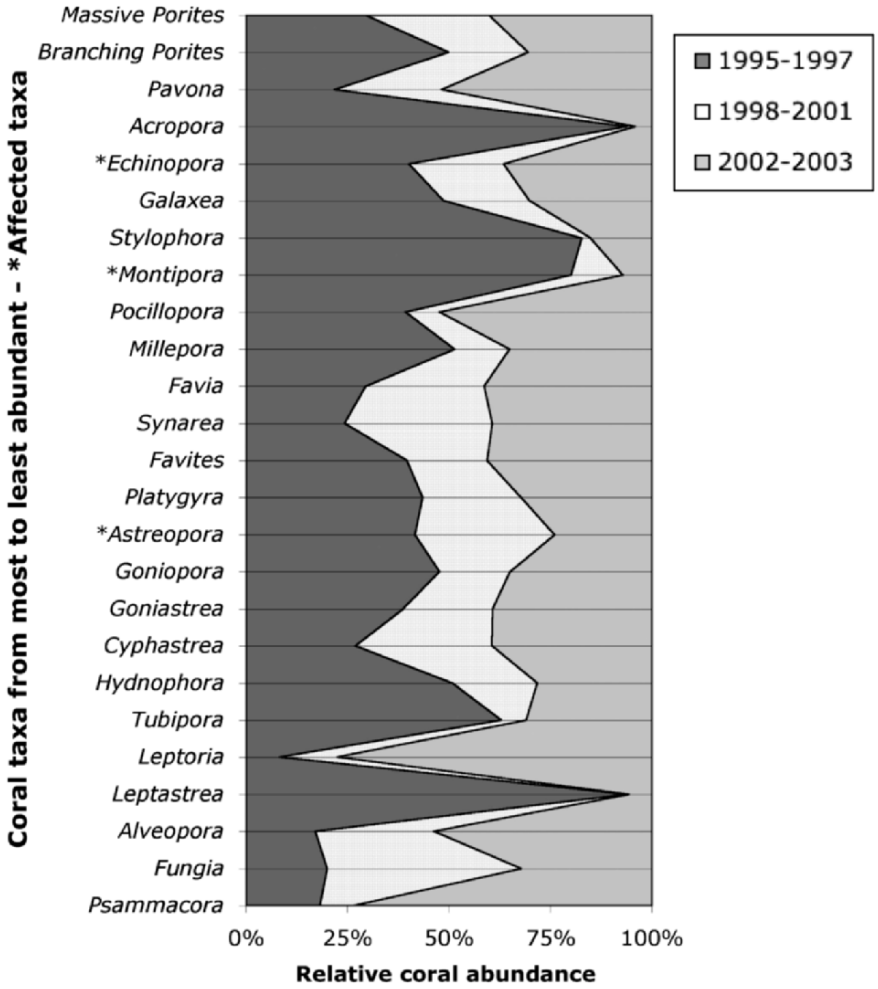

Figure 6. Changes in the relative coral assemblage 1995–2003. Coral bleaching occurred in 1998–2001, and the disease outbreak was in early January–February 2002. Affected genera are those that died but a few other genera were affected and did not die.

SEM micrographs

Fungal filaments were observed in affected but not healthy-appearing samples (Fig. 4). Some fungal filaments appeared to be passing through the skeleton but the direction of growth was difficult to ascertain.

Ecological consequences

The consequences of this disease were not as great as the coral-bleaching event in 1997–1998 (Fig. 5A), but they did reduce recovery rates of the taxa most affected by the 1998 event. The affected taxa were not a large part of the total coral cover so there was not a significant change in total cover and its recovery. *Echinopora* and *Astreopora* often exhibited partial mortality, which appears to have resulted in little change over the study period (Fig. 6). In the case of *Montipora*, however, mortality from this disease combined with that from the 1998 bleaching event reduced its abundance below the level of reliable detection by our sampling. Because *Astreopora* was not an abundant genus and the estimates of its abundance have high variability, it was difficult to detect the effect of this disease on its abundance (Figs 5 and 6).

Discussion

The gross signs of this disease are not nearly as striking as coral bleaching since the affected coral tissue becomes dull rather than bright white or fluorescent (McClanahan et al., 2001), and often is hidden by mucus and a white dust as the coral dies. Unlike bleaching, which affects most taxa (McClanahan et al., 2001), this disease affected few taxa on these reefs. Although the appearance of patches of bare skeleton seems similar to white plague disease of massive corals (Pantos et al., 2003), which has not been recorded in the western Indian Ocean, the changes in coloration of the live tissue and results of the histopathological examinations do not support this diagnosis (Richardson et al., 1998). Unusual aspects of this syndrome include rapid mortality but coating of the skeleton with fine calcareous dust. The dust might result from a boring sponge or other organism that decays the skeleton, but microscopic observations did not identify borers apart from fungi. Unlike microbial band-forming diseases, there was no band formation. There was a very sharp and distinct line between live coral and bare skeleton; discoloration along the margin was slight, but evident.

Histopathological examinations revealed extensive necrosis and lysis of tissues in *Montipora aequituberculata*, *Astreopora myriophthalma*, and *Echinopora gemmacea*. The sharp delineation between lesions and apparently healthy areas observed with the unaided eye was also evident at the light microscopic level. In addition, the characteristic large tentacles of *Gonoipora stokesi* were often missing. Lesions occurred in *Porites lutea* at significantly lower frequencies, perhaps suggesting an ability to resist the disease. The cause of lesions remains uncertain although bacteria and fungi were found at differing levels among the coral genera. The extensive tissue lysis observed in many specimens support the presence of toxin-producing bacteria. Some fungal filaments were observed in *M. aequituberculata*, *P. lutea*, and *E. gemmacea*. The last was the only species examined that had dense fungal mats along its surface and many fungi within skeletal spaces. It is unknown whether fungal mats on the surface were washed away during the histology process in other species. Further, decalcification in the histology process eliminated an important component of the coral specimen. Whether the fungi and bacteria present in the tissues in this study were primary or secondary invaders remains to be determined. They were, however, more common in the corals that died than in those that were affected but did not die. Current studies to embed coral specimens in resin without decalcification may shed light on the role of fungi and bacteria in the disease process.

The gross and microscopic changes observed in these corals do not indicate a microbial invasion and consumption of tissue that migrates into living tissue just at the tissue margin, as is found in white plague (Richardson et al., 1998). Rather, an alteration of the normal protective epidermis might have occurred, either from an abiotic change in water quality or a pathogenic microorganism, leading to proliferation of bacteria, fungi, and sloughing of necrotic tissue. The disappearance of the syndrome after the change in the monsoons in April 2002 indicated it was not a persistent pathogen, but perhaps seasonal and rare. Unfortunately, collection of samples for isolation and identification of microorganisms could not be attempted at these field sites. Even when this has been done, difficulties in microbial characterization arise (Ritchie et al., 2001). For example, the bacterium *Aurantimonas coralicida* has been identified as a cause of white plague disease (Denner et al., 2003) but was not identified in the white plague samples of Pantos and colleagues (2003), who reported that the microbial fauna was complex and similar to black-band disease.

Water temperatures prior to this event were not very different from other years. The one unusual aspect was the lack of a strong northeast monsoon, which often cools the water in January. The weak monsoon did not, however, result in unusually warm water, in contrast to the bleaching year of 1998. Observations of coral death in northern Kenya corresponded with a red tide (J. Church, personal communication). It is unlikely, however, that the red tide caused the coral death as no red tides were observed in southern Kenya. There was a 3–4 days' bloom or current drift of gelatinous zooplankton in southern Kenya, but it was not associated with rich phytoplankton. It is possible the disease was caused by water chemistry changes, but the taxon-specific response is difficult to explain since it was not the fast-growing taxa that were most affected.

Diseases often have complex etiologies and it can be difficult to determine whether primary or secondary pathogen(s) are involved, especially when abiotic stressors could increase the potential for susceptibility to infection by pathogens. The most likely explanations for the mortalities, based on field, histology, and SEM observations are: (1) the affected species were infected by a pathogenic microorganism (primary pathogen) and tissues were invaded by fungi (secondary pathogen), (2) coral tissue weakened by changes in water quality was invaded by a foreign fungus possibly associated with the northeast monsoon water mass, (3) the observed fungal hyphae are extensions of endolithic fungi that coexist with corals but the usual coexistence was tipped in favor of the fungi. At present, it is difficult to distinguish among these alternatives. Recent observations on coral-fungi associations (Le Campion-Alsumard et al., 1995a; Bentis et al., 2000) suggest the latter two hypotheses are likely. Endolithic fungi living in close association with coral are a mix of species found on land and those unique to the sea (Raghukumar & Raghukumar, 1991; Smith et al., 1998; Ravindran et al., 2001). Some are solely endolithic and live in carbonate substrates without living coral and some appear to require living coral (Raghukumar & Raghukumar, 1991; Ravindran et al., 2001). The interaction between these fungi and endolithic algae create the black band formations often seen in coral skeletons (Priess et al., 2000). SEM observations indicate that fungal hyphae occasionally penetrate the coral skeleton and enter the coral tissue (Le Campion-Alsumard et al., 1995b; Bentis et al., 2000). Corals respond or defend themselves by calcifying over the pore created by the hyphae, so that most hyphae observed in healthy corals are nob-like protuberances, and only a few have pores that indicate penetration into the coral tissue (Le Campion-Alsumard et al., 1995b; Bentis et al., 2000). Thus, corals and endolithic fungi may largely live commensally, but the relationship could range from symbiotic to saprophytic. Our SEM observations suggest some instances of penetration of the hyphae through the coral skeleton but their direction of movement is not obvious: hyphae could have been penetrating from either the tissue or from beneath the skeleton. We did not observe the nob-like protuberances that indicate a healthy calcifying coral resisting invasion from the underside, which may indicate that penetration was from the tissue to the skeleton. This would support the hypothesis that the fungus was a foreign invader. If the corals had stopped calcifying, it would be difficult to know the direction of the invasion. Further support for the foreign-invader hypothesis is that most histological evidence for fungi was in species that died from the disease and less from those that displayed signs of morbidity but did not die. This suggests that fungi invaded once the coral was weak and increased the chances for mortality. Further, in the case of *Porites lutea*, the lesions appeared to start at the mouth, suggesting some invasion from the water or food.

Distinguishing among hypotheses is difficult, similar to many complex disease problems. One way to improve understanding and distinguish cause and effect would be to examine coral skeletons as described by Bentis et al. (2000) for evidence of pores penetrated by fungal hyphae. The frequency of these pores in the protuberances should indicate whether the coral was calcifying when the fungus invaded and whether the fungus invaded from beneath the skeleton during a temporary hiatus in calcification when the coral was unhealthy or after the coral died. The frequency of pores with and without protuberences should change depending on the succession of invading microbes, and may be a direct measure of the health of corals. Given the close relationship between coral and fungi, it is likely that fungi consume corals that are ill or dying prior to either recovery or death by other means. Consequently, whether the coral death is caused by an imbalance in the coral-fungi relationship or if an external environmental factor created suboptimal conditions for corals, which then allowed fungi to invade and kill the coral, is difficult to resolve.

The ability of corals to resist or reverse cellular damage by microorganisms, as indicated by the recovery of *Porties lutea* and *Goniopora stokesi*, is poorly understood, especially since the characterized infectious scleractinian diseases usually result in complete loss of tissue, with subsequent fouling and bioerosion of the carbonate (Peters, 1997; Richardson, 1998). In this study, the histopathological examinations suggest that the oral surface tissues were killed and lysed (giving the

appearance of a mucus covering). Perhaps the polyps and coenosarc tissue regenerated from cells deep in the skeleton lining the gastrovascular canals between or within polyps (e.g. Riegl & Pillar, 2001). Conditions in this region of the corallum may not have been favorable for microbes or perhaps the coral cells were protected from toxic substances or poor water quality. The results of this study provide additional insights into the complexity of coral diseases and the importance of field assessments and laboratory analyses using many tools.

Acknowledgments

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Coral mortality, recovery and reef degradation at Mexico Rocks Patch Reef Complex, Northern Belize, Central America: 1995–1997

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Key words: biotic phase shift

Abstract

The 1995 coral bleaching event in the western Caribbean was the first reported episode that significantly affected the Belize barrier and lagoonal patch reefs. Bleaching was attributed to a 2 mo period of warm water temperatures above 30 °C. Near Ambergris Caye, barrier and patch reefs experienced up to 50% bleaching. At Mexico Rocks patch reef complex, the bleaching resulted in changes in reef health, community, and physical structure. Prior to the hyperthermal episode, patch reef surface area consisted of 47% healthy framework coral coverage, 12% secondarily colonized biotic coverage, 35% dead coral surfaces that were degraded by biological activity and physical erosion, and 6% cavities. six months after bleaching, most corals had regained their color, but, owing to coral mortality, areas of surface degradation had increased to an average 49% ($p = 0.029$ based on Kruskal–Wallis analyses). Eighteen months after bleaching, degraded surface areas expanded to 53% ($p = 0.0366$). Although re-coloring indicates rapid recovery for surviving corals, the persistence in dead coral surfaces suggests that reef skeletal structure recovery lags behind that of individual corals. Initial results of framework measurements indicate that bleaching events may result in an 'imbalance' in the carbonate production rate of coral reefs and produce mass wasting of the skeletal structure. Remapping of reef skeletal structure should establish quantitative measures for the long-term effects of bleaching on patch reef frameworks.

Introduction

Coral bleaching as a result of higher than average water temperatures commonly has been associated with El Nino/Southern Oscillation events as reported in the Pacific in 1982–1983, 1987, and 1998 (Glynn, 1988a, 1993; Viets, 1998). Elevated sea surface temperatures in the western Atlantic from August through October 1995 also produced widespread bleaching of corals from the Belize barrier and lagoonal patch reefs (Holden, 1995; CARICOMP, 1997). This event marked the first documented coral bleaching in this area (Stout, 1995). The Mexico Rocks patch reef complex, located 0.3 km seaward of Ambergris Caye in northern Belize, was severely affected by this thermal episode, when surface water temperatures in the shallow back-reef area in northern Belize increased to 32–34 °C (Sprowls, 1995). Aerial and underwater surveys of the bleaching event indicated that as much as 50% of the corals were bleached both at Mexico Rocks and on the barrier reef (CARICOMP, 1997). This paper presents the effects of the 1995 bleaching episode at the Mexico Rocks patch reefs, summarizes short-term coral recovery since that time, and describes long-term effects on patch reef skeletal structure.

Bleaching occurs when stony corals lose or expel all or a portion of their endosymbionts (zooxanthellae). Several factors have been impli-

cated for this disruptive process, including disease (Kushmaro et al., 1966; Gleason & Wellington, 1993; Ritchie et al., 1993), increased UV radiation flux (Jokiel & York, 1984), hyposalinity (Goodbody, 1961; Goreau, 1964), increased sediment flux (Acevedo & Goenaga, 1986), pollution (Neff & Anderson, 1981), and temperature increases, some of which are possibly associated with global warming (Jokiel & Coles, 1990; Glynn, 1991, 1993; Smith & Buddemeier, 1992).

The ability of corals to recover their zooxanthellae after bleaching appears to be species-specific and related to their susceptibility to increases in water temperature, and recovery period can range from months to years (Hays & Bush, 1990; Holden, 1995). Research on bleached and then recovered *Montastrea annularis* in reefs in the Cayman Islands, for example, indicates that healing is gradual, and involves acquisition of a new population of zooxanthellae and restoration of their densities to normal, non-bleached levels (Hays & Bush, 1990). Additional consequences of bleaching include decreased skeletal growth, repressed gonad growth and reproduction, increased predation pressure on surviving corals, and increased mortality (Jokiel & Coles, 1977, 1984; Glynn, 1988b, 1990, 1993; Hughes, 1989; Brown & Suharsono, 1990; Goreau & MacFarlane, 1990; Szmant & Gassman, 1990). Occasional reports of framework deterioration have also been reported (Glynn, 1988b, 1993; Eakin, 1991).

Study area

Mexico Rocks patch reef complex is located on the outer shelf platform offshore of northern Belize, about 0.3 km seaward of Ambergris Caye, and 0.4 km to the lee of the platform-margin barrier reef. Dimensions of the complex are approximately 1.7 km in length and 0.5 km in width. It has been under consideration for preserve status by the Belize government, and has been the site of baseline research by the authors since 1988 (Mazzullo et al., 1992, 1993; McHenry, 1996; Burke et al., 1998). The complex includes approximately 100 individual patch reefs, which consist predominantly of the *Montastrea annularis* complex of coral heads that range in area from approximately 4–400 m^2. The largest of these reefs are about 2.1 m high and have grown to within 0.5 m of mean sea level. The patch reefs have grown atop and on the flanks of a narrow, northeast-trending ridge of karsted Pleistocene limestone. Growth was initiated during the Flandrian transgression at about 3.5 Ka ago (Mazzullo et al., 1992, 1993; Burke et al., 1998).

Water depths immediately around the complex and between patch reefs average 2.7 m, and increase to about 4 m in a seaward direction before shallowing to about 0.9 m toward the barrier reef flat. Temperature, salinity, and pH were recorded at least once a year within and around the complex over the period from 1988 to 1998. Salinity of the water is constant at 38‰, and daytime pH ranges from 8.0 to 8.4. Average daily surface ocean temperatures range seasonally from 27 to 29 °C. The semi-diurnal tidal range is less than 0.5 m and typical wave energy, which is qualitatively characterized as moderate (except during storms or unusually calm weather), is driven by onshore and seasonal easterly trade winds and, to a lesser extent, by tides. Some wave energy is input through passes in the barrier reef.

Methods

Twenty-three patch reefs ranging from 4 to 370 m^2 were geologically mapped and biologically surveyed (using line transect and area measurements) in 1990 for determination of coral–algal coverage, percentage of dead coral (herein called areas of degradation), percentage of cavities, and both linear and vertical dimensions of each patch reef framework (cf. Burke et al., 1998 for specific field methods and survey results). A rope line, calibrated at meter intervals, was placed along the long axis of each of the 23 selected reefs. At each meter section, both area measurements of biotic coverage, degradation and cavities, and the biota beneath the rope line were recorded on underwater slates for the length of each patch reef. A steel reinforcement bar calibrated at 0.3 m intervals was used for field measurements of horizontal and vertical dimensions at each meter interval along the rope line. Horizontal measurements included the width of each meter section along the length of the patch reef in meters. At each 1 m interval, vertical measurements were taken at the center of

the reef, and at least two locations perpendicular to the long axis. This resulted in a minimum of three vertical measurements for each meter section across the top and flanks of each patch reef. To assure good correspondence between reef topography and contour mapping, wide or cavernous reefs required additional height measurements. All dimensional measurements from each patch reef were used to construct contour maps of each patch that also serve as base maps for annual monitoring of reef health. Yearly assessment of coral–algal coverage, percentage of dead coral, percentage of cavities on the reef surface, and changes in dimensions of the patch reefs, can be compared to these original base maps for each of the 23 patch reefs. This field technique provides a method to monitor the health of reef skeletal structure. An estimate of framework erosion can be determined by comparing original base maps to annual assessments of surface area degradation and cavities present as well as to vertical distance below mean sea level to the tops of the colonies. These data can be useful for monitoring long term, temporal changes in the skeletal framework and coral coverage on patch reefs, or any section of any reef.

Results and discussion

Pre-bleached parameters

Prior to the 1995 hyperthermal episode, patch reef surface area consisted of 47% healthy stony coral coverage, 10% soft coral/sponge coverage, 2% algae, 35% dead coral surfaces that were degraded by biological activity and physical erosion, and 6% cavities (Fig. 1). On the 23 measured patch reefs, the head corals *Montastrea annularis* (*sensu strictu*) composed 83%, and *Diploria* spp. and *Dichocoenia* sp. together composed 1% of total area of living reef-framework biota (coral species coverage). Other species of *Montastrea* (e.g., *faveolata*, *franksi*, and *cavernosa*) were observed in the complex, but were localized, and did not constitute a noteworthy portion of the measured patch reefs or of the complex. The remaining 16% of corals consisted of *Agaricia* spp., *Porites* spp., *Acropora* spp., and other branching scleractinids; and reef-dwelling *Millepora* spp.

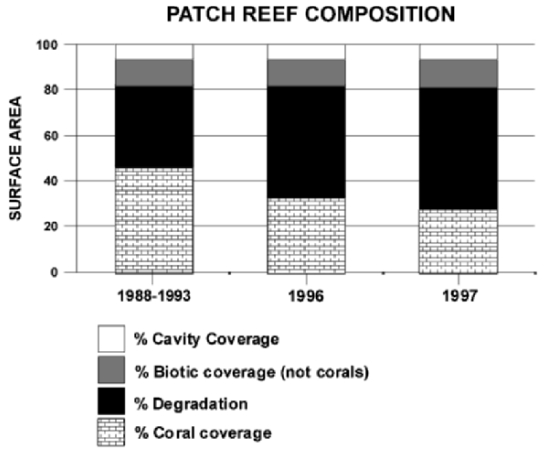

Figure 1. Patch reef composition. Weighted percentage by area of coral and non-coral biotic coverage, degradation, and surface cavities at the Mexico Rocks patch reef complex, 1988–1997.

Degraded areas on the framework resulting from biological and physical erosion averaged 35% of measured reef surface area (Fig. 1). Additional coral species (e.g., *Agaracia* spp., *Acropora* spp., and *Porities* spp.) and, locally, calcareous algae (e.g., *Halimeda* spp. and *Amphiroa* spp.), filamentous (turf) algae, and macro algae (e.g., *Padina* and *Turbinaria*) or encrusting and boring sponges, colonized these areas of dead coral, thereby increasing species richness on the patch reefs and comprising 12% of biotic coverage (Mazzullo et al., 1992, 1993; McHenry, 1996; Burke et al., 1998). The distribution of non-scleractinid coral biota was controlled by the amount and distribution of surface degradation and cavities (Burke et al., 1998). Commonly, localized growths of non-calcifying macro algae, turf algae, and encrusting sponges were restricted to large, degraded patch reefs, and were rarely found growing on medium or small patch reefs with little degradation. Calcareous algae and non-encrusting sponges grew in degraded regions among lobes of corals and skeletal cavities.

Cavities on the patch reefs averaged 6% of the complex (Fig. 1). Most of the medium and small clusters of head corals contained few cavities; larger patch reefs generally contained larger cavities. Because of the dominance of head corals, the reefs were clustered domes that had coalesced over time and grown to within 0.5 m of sea level. No significant changes in the geometric shapes of the

patch reefs, heights below mean sea level, species coverage, degraded areas, and extent of cavities were noted from 1988 to 1993 (Burke et al., 1998). In fact, the 23 patch reefs in the complex were easily identifiable from year to year based upon these attributes.

Post-bleaching parameters – 1996

Increase in degradation, shift in species distribution, and presence of diseased corals

The patch reefs were re-surveyed in March 1996, 6 mo after the 1995 bleaching event. Evidence of active bleaching was minimal: less than 1% of the corals were bleached. Most of the previously whitened corals had regained their normal color, and presumably, their population of zooxanthellae. Rapid recovery of surviving corals after the bleaching event therefore is indicated.

To determine the extent of coral survival, we re-measured the area of degraded reef framework. Dead reef surfaces had increased on average to 49% of the reef frameworks (Figs 1 and 2). This significant increase ($p = 0.029$ based on Kruskal–Wallis analyses) was the result of coral mortality and indicates that the 1995 Caribbean bleaching event resulted in significant coral mortality on these patch reefs within 6 months. Most of these 'new' zones of degradation were already colonized by algae, a phenomena that is noted by many Pacific reef workers as a post-bleaching ramification (cf. Jokiel & Coles, 1977; Glynn, 1988a, 1993; Hughes, 1989; Brown & Suharsono, 1990; Goreau & Macfarlane, 1990; Szmant & Gassman, 1990; McCook, 1999).

Increase in standing crop of algae indicates a phase shift in biotic distribution on the reefs. Most of the degraded surfaces at Mexico Rocks had been colonized extensively, and in some cases, entire reefs were nearly overgrown, by noncalcifying turf algae. To further complicate recovery, macro-algae, including intertwining mats of *Caulerpa racemosa* and meadows of *Turbinaria* spp. and *Padina* spp. covered the patch reef surfaces. Before 1996 these algae were restricted to large, degraded portions of reefs in the complex. In 1996, nearly all the patch reefs supported macroalgae, and in some cases, entire reefs were nearly overgrown by *Caulerpa racemosa*. This increase in macroalgal abundance may have resulted from coral mortality, corals weakened by bleaching with inhibited natural chemical and physical defense systems (cf. Lang, 1973), and lack of algal herbivory. As a consequence, algal standing crop increased, and resulted in a dramatic phase shift in reef biotic distribution (cf. Jokiel & Coles, 1977; Glynn, 1988a, 1993; Hughes, 1989; 1994; Brown & Suharsono, 1990; Goreau & Macfarlane, 1990; Szmant & Gassman, 1990; McCook, 1999).

Coral bleaching has been implicated in the suppression of normal coral physiology including carbonate secretion (Glynn, 1993). The increase in

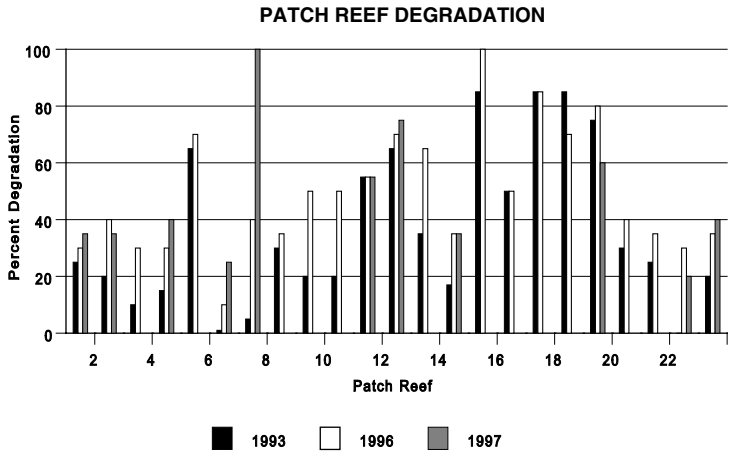

Figure 2. Patch reef degradation. Percentage of patch reef surface area degraded in 1988–1993, 1996, and 1997. Solid black bar is area degraded prior to bleaching (1993). Solid white bar is area degraded in 1996, 6 months after bleaching. Gray bar is area degraded in 1997, 18 mo after bleaching. The degraded surface area on the patch reefs increased significantly after the 1995 bleaching event.

dead reef surfaces may reflect these physiological complications. For example, corals weakened by bleaching may be unable to ward off pathogens or disease epizooids that ultimately lead to coral mortality. As noted by Glynn (1993, p. 5), increases in coral diseases may be a result of stressors placed on corals by bleaching.

By March, 1996, previously unrecorded coral diseases such as White Plague and Pox were present on the reefs, but were sufficiently uncommon to warrant a category on survey transects. Evidence of white band and plague diseases were present in the form of fresh white spotting of coral surfaces that were devoid of living corals. Black band disease was also present, but has been a persistent, contained disease in the complex for many years.

Post-bleaching – 1997

Increase in degradation and change in patch reef geometry

The patch reefs were again resurveyed in 1997, 18 mo after the bleaching event. At this time, less than 1% of the corals, including the dominant frame builder, *Montastrea annularis* (*sensu strictu*), displayed signs of active bleaching. An 'apparent' shift in morphotypes to *M. franksi* was noted but not quantified on several reefs. Localized areas of diseased corals were present in the complex as in the previous year.

The average amount of surface degraded area, however, had increased from 49% to 53% in 1996. This increase in degraded areas from 1996 to 1997 (Fig. 1) is statistically significant ($p = 0.0366$), and suggests that coral mortality continued to occur throughout 1996–1997. Based upon these results, several implications can be made. First, re-coloration of corals is only a partial measure of reef recovery and occurs rapidly after waters cool. Secondly, recovery of the reef framework lags behind that of individual corals, and may be at risk because fewer corals are producing calcium carbonate due to coral mortality. Evidence for framework decline was present by 1997.

Patch Reefs 15 and 7 (87.7 and 4.2 m^2 in area, respectively), which are located in different areas of the complex, had collapsed and all but disappeared (Fig. 2). A less dramatic, but typical example of skeletal structure collapse is patch reef 13, located in the northern portion of the complex. In 1993, mapping of this patch reef indicated that the total volume of the framework was 26 m^3. Remapping of the patch reef in 1997 indicated that the total volume of the reef was 24 m^3 which represents an approximate loss of about 2 m^3 of carbonate. This decrease in volume was attributable to reduction in the height of the reef below mean sea level. In essence, the large domed *Montastrea annularis* head that comprised the reef framework had collapsed, and produced an erratically cratered reef topography. Similar signs of mass wasting were present throughout the complex such that patch reefs easily recognizable through geometric shape by the authors in previous years were unrecognizable except by map location in 1997. A study to quantify the significance of this carbonate loss is underway.

Conclusion

Although reports on global coral health suggest that coral reefs remotely located away from centers of human population are not deteriorating (Pennisi, 1997), near shore reefs like Mexico Rocks are more typical of the patch reefs associated with increasing eco-tourism, harvesting, and onshore development. Conditions here are generally well disposed for reef growth; however, lagoonal patch reefs at Mexico Rocks are subjected to more variable environmental conditions and anthropogenic stresses because of their proximity to shore than are remotely located reefs. These shallow water patch reefs were among the first to succumb to bleaching, and their recovery time was slower than those corals that comprise the barrier reef (CARICOMP, 1997).

At Mexico Rocks, the sequence of post-bleaching events that was observed on the patch reefs includes (1) significant increase in coral mortality as indicated by dead coral surface coverage and diseased corals, (2) phase shift in biotic distribution, and (3) initial deterioration of reef framework. Re-coloration of surviving corals developed soon after waters began to cool. Deterioration of reef frameworks, however, may be a long-term process.

Based on radiocarbon age dates, patch reefs at Mexico Rocks have grown and diversified for

hundreds of years (Burke et al., 1998). In contrast, only a few years were required to initiate reef deterioration after living reefs were subjected to a severe bleaching event, the predictable results of which were coral mortality and algal increase. When corals do not produce calcium carbonate exceeding or in equilibrium with reef erosional processes, reef skeletal structures may undergo mass wasting by biological and physical degradation. Analogous reef skeletal structure demise was observed in the Pacific as a result of the 1982–1983 El Nino by Glynn (1988b, 1993) and Eakin (1991). Further study of these patch reefs is necessary to determine the significance and permanency of framework mass wasting.

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Synoptic water clarity assessment in the Florida Keys using diffuse attenuation coefficient estimated from Landsat imagery

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Key words: remote sensing, satellite, water clarity, coral

Abstract

The diffuse attenuation coefficient, K (m^{-1}), is a measure of the effective attenuation of light in the water column. It characterizes water clarity and is used as a proxy for water quality. Mapping of shallow water benthic habitats using optical means, including daytime visible satellite imagery, requires knowledge of K to correct for water column effects such as light absorption and scattering. Traditionally, K is derived from imagery using *a priori* knowledge of bottom types at different depths and specific locations, and assuming that light attenuates exponentially with depth. This technique is applied to three Landsat 7 satellite images (February, May, and July 2000) from the Florida Keys Reef Tract between Key Largo and Key West. Interpolated depth data, initially from NOAA vector chart data, with uncertainties of ± 0.5 m, were draped over 30 m spatial resolution Landsat satellite imagery. K was derived for 27 sites where bright sand could be observed at depths between 3 and 20 m. The blue and green bands (Landsat bands 1 and 2 at 450–520 nm and 520–600 nm, respectively) provided K values consistent with time and location. Average K values for bands 1 and 2, respectively, were 0.029 and 0.043 m^{-1} (Lower Keys), 0.050 and 0.072 m^{-1} (Middle Keys), and 0.063 and 0.082 m^{-1} (Upper Keys). The red band (band 3, 630–690 nm) provided more ambiguous and erroneous results with several negative K values, attributed primarily to three factors: use of a simple atmospheric correction, the high absorption and rapid attenuation of red light in water, and low radiometric sensitivity of the Landsat ETM+ sensor. Despite the fact that these observations were a snapshot in time, trends were observed for the Upper, Middle, and Lower Keys, possibly due to the influence of more turbid Florida Bay waters.

Introduction

Water quality is important for the reef ecosystems of the Florida Keys (LaPointe & Matzie, 1996; Szmant & Forrester, 1996). Levels can fluctuate with the presence of various inputs, including nutrients, chlorophyll, and phytoplankton. Declining water quality has been proposed as the major cause for the increase in disease visible on reefs of the Florida Keys (Lipp et al., 2002; Patterson et al., 2002) as well as the phase shift from coral to algal communities (LaPointe, 1997). Efforts to study water quality parameters in the Florida Keys include seasonal *in situ* data collection via the Environmental Protection Agency's (EPA) Water Quality Monitoring Project (Boyer et al., 2000) and the SEAKEYS program buoy data (Ogden et al., 1994). It is now apparent that water quality and clarity are highly variable, both seasonally and spatially, within the three major geologic zones of the Florida Keys (Upper, Middle, and Lower; Smith, 1994; Szmant & Forrester,

1996; Smith, 1998). The highest nutrient concentrations seem to be in the Middle Keys during the summer months (LaPointe & Clark, 1992). Yet to date there is no systematic synoptic estimate of water quality for the Florida Keys at fine resolution.

Remote sensing by satellites can help by providing rapid and repeated assessments over large, synoptic scales. In this paper, we explore the possibility of assessing water quality parameters over the Florida Keys with the Landsat 7 Enhanced Thematic Mapper Plus (ETM+).

The diffuse attenuation coefficient, K (m^{-1}) is an apparent optical property (i.e. varies with illumination conditions). It describes the effective attenuation of light in the water column, and hence is a measure of water clarity. The derivation of K is based on the assumption that light attenuates exponentially with depth (Kirk, 2003). To classify shallow benthic habitats using remotely sensed imagery, K is required (Maritorena et al., 1994). However, it has seldom been measured concurrently with satellite overpasses. K, or the ratio of K in different spectral bands, has been traditionally derived directly from satellite imagery over similar bottom types (e.g. sand) located at different depths (Maritorena, 1996). For two pixels at different depths, z_1 and z_2, K is estimated as

$$K = \ln [L_w(z_1)/L_w(z_2)]/[2(z_2 - z_1)]. \quad (1)$$

Here, L_w is the water-leaving radiance, i.e. upward radiance from the water column measured just above the surface (surface reflected light is excluded). A factor of 2 approximates the attenuation of light as photons travel in both the downward and upward directions (Maritorena et al., 1994).

We derived K at several sites with Landsat satellite imagery to study spatial and temporal variations of this parameter in the Florida Keys Reef Tract. The technique was applied to bands 1 (blue, 450–520 nm), 2 (green, 520–600 nm), and 3 (red, 630–690 nm) of the Landsat 7 ETM+ imagery. Three images were acquired for 5 February, 27 May, and 14 July of 2000 between Key Largo and Key West. Specifically, this study addresses the variation of K throughout the Florida Keys' three geologic zones: Upper, Middle, and Lower Keys, for the winter (February), spring (May), and summer (July) 2000 season.

Methods

Bands 1–3 (within the visible light range) of the ETM+ sensor on board Landsat 7 were radiometrically calibrated from digital number (DN) to total radiance (L). A very simple atmospheric correction was effected by subtracting the total radiance obtained in each band over deep water from the total radiance in the same band over adjacent shallow water areas. While this overcorrects the atmosphere's color and renders an underestimate of the water-leaving radiance, this artifact is ignored because water-leaving radiance over shallow water with benthic sand cover is typically an order of magnitude larger than over deep clear water. We assume that the result is L_w^* ($= L_w \cdot t$, where t is the diffuse transmittance from water to the sensor; Lyzenga, 1981) and that this can be used in Equation (1) in lieu of L_w. The bathymetry data, originally from NOAA vector chart data at 1 and 5 m depth intervals, were interpolated to 1 m depth intervals and 30 m spatial resolution. The depth data were then registered to and draped over the 30 m Landsat data. For each of the three Landsat bands, L_w^* values were taken over sand for 27 sites throughout the image (Fig. 1). Each of these sites provided sand patches at various depths (z_1, z_2, ...) so that an estimation of K could be derived (Equation 1). The deepwater pixels were chosen as close as possible to the 27 sites.

Results and discussion

The K values obtained for the red band were often negative and thus were invalid. This error can be attributed primarily to three factors: the use of a simple atmospheric correction (which overcorrects each band), the high absorption and rapid attenuation of red light in water, and the low radiometric sensitivity of the Landsat 7 ETM+ sensor. The results from the blue and green bands were far more consistent, although some negative values also occurred (a total of five values in the blue and three in the green of a total 81 K values per band).

Average K values were lowest (clearest water) for the Lower Keys ($K_{b1} = 0.029\ m^{-1}$ and $K_{b2} = 0.043\ m^{-1}$), followed by the Middle Keys ($K_{b1} = 0.050\ m^{-1}$ and $K_{b2} = 0.072\ m^{-1}$, excluding

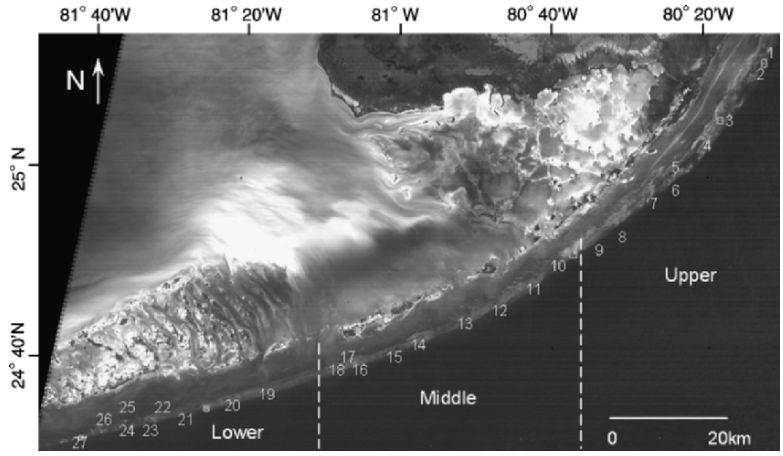

Figure 1. Landsat image of the Florida Keys, displaying the location of the 27 sites in the three major geologic zones (delineated by dashed lines) within which the diffuse attenuation coefficient (K) was estimated.

the May image), and were highest in the Upper Keys ($K_{b1} = 0.063$ m^{-1} and $K_{b2} = 0.082$ m^{-1}) (Fig. 2).

The May Middle Keys image exhibited K values 4× (band 1) and 2× (band 2) the mean value derived from the other images. If the K values from this image were averaged with the K values from February and July for the Middle Keys, the new value ($K_{b1} = 0.0943$ m^{-1} and $K_{b2} = 0.0995$ m^{-1}) would be higher (less clear water) than the Upper Keys. These patterns suggest that the Middle Keys, which are typically considered the most turbid due to more direct influence from Florida Bay waters (Porter et al., 2002), may also sometimes have clearer water relative to the Upper Keys.

This high K anomaly was mirrored in the ocean color data from the Sea-viewing Wide Field-of-View Sensor (SeaWiFS; McClain et al., 1998), which showed that the color-rich patterns in the Middle Keys also occurred from May to June in 2001, 2002, and 2003. Since the SeaWiFS signal is dominated by chlorophyll *a* or colored dissolved organic matter, the result agrees with that of LaPointe & Clark (1992), who found the highest chlorophyll *a* levels during the summer for this same geographic region. However, it is difficult to derive conclusions about seasonality in K from only the three Landsat images examined here, and similar processing needs to be repeated.

For benthic classification, concurrent measurements of K are required to optimize the correction of optical effects due to the water column (Mumby et al., 1998). Palandro et al. (unpublished data) compared benthic classification results from Landsat with *in situ* data taken by the Coral Reef Monitoring Project (CRMP) (Porter et al., 2002) for 1996, 1998, 2000, and 2002 for five reefs (Carysfort, Grecian Rocks, Alligator, Looe Key, and Eastern Sambo Reefs). Overall percent decline in coral cover was 43.51% (Landsat, percent 'coral-habitat' pixels) and 42.45% (CRMP, coral cover data) between 1996 and 2002. The agreement in percent loss values between these studies was made possible because derived K values were used to correct the Landsat imagery. These results will be presented in detail in a subsequent paper.

Conclusion

We estimated the diffuse attenuation coefficient in water, K (m^{-1}), in the Florida Keys from Landsat imagery for February, May, and July 2000. Results from two visible bands (centered at 485 and 560 nm, respectively) show generally consistent behavior for the Lower, Middle, and Upper Keys. A more rigorous atmospheric correction would help derive better estimates of K. Observations of large-scale variations of K, in the blue and green bands, at the three times suggest that snapshots in water quality somewhat reflect the expected geographical trends in water quality variations within the Florida Keys seascape. However, it is not

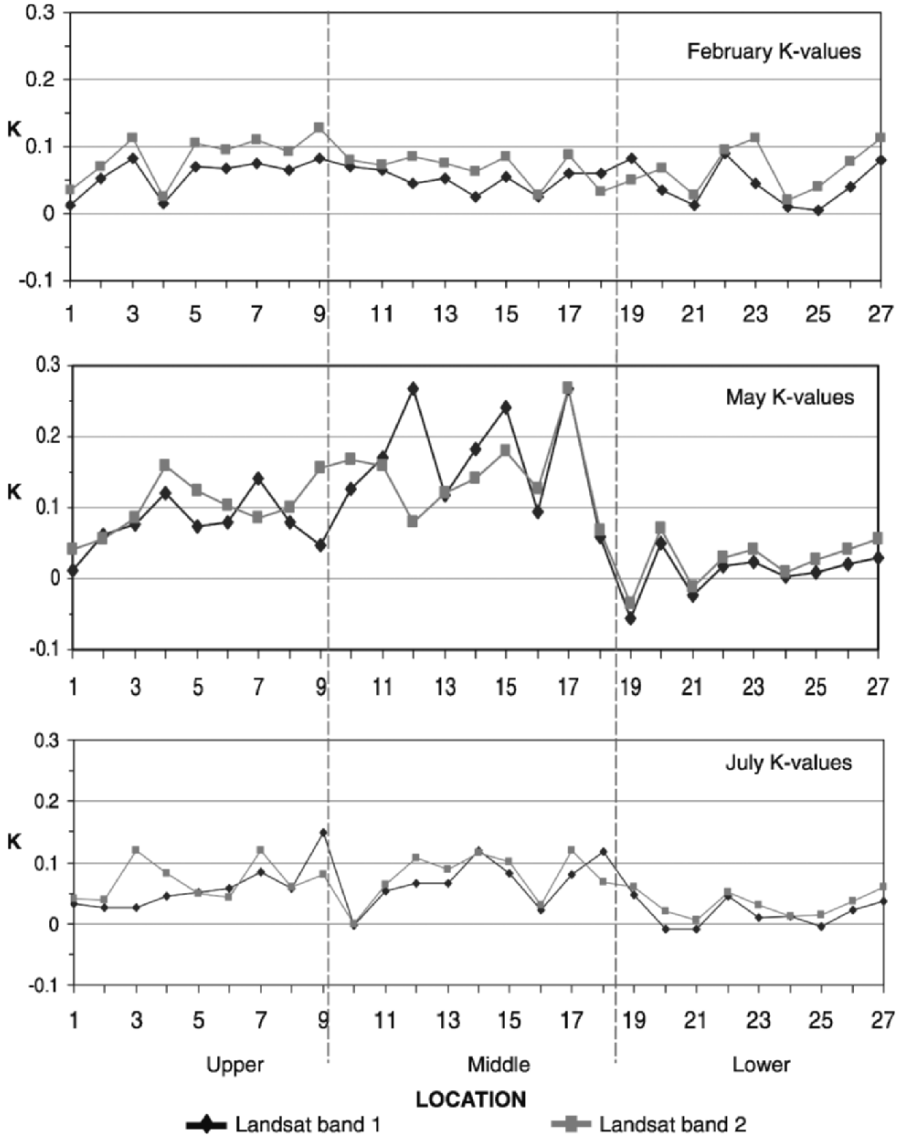

Figure 2. Graphs displaying K values (m^{-1}) derived from Landsat bands one (blue) and two (green) for each of the three images studied. The location values refer to the sites displayed in Figure 1.

possible to make concrete statements about seasonal changes or trends based on this sparse dataset.

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Part VIII
Ecology

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Distribution of sea anemones (Cnidaria, Actiniaria) in Korea analyzed by environmental clustering

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Key words: geospatial clustering, LOICZView, biogeography, Anthozoa

Abstract

Using environmental data and the geospatial clustering tools LOICZView and DISCO, we empirically tested the postulated existence and boundaries of four biogeographic regions in the southern part of the Korean peninsula. Environmental variables used included wind speed, sea surface temperature (SST), salinity, tidal amplitude, and the chlorophyll spectral signal. Our analysis confirmed the existence of four biogeographic regions, but the details of the borders between them differ from those previously postulated. Specimen-level distribution records of intertidal sea anemones were mapped; their distribution relative to the environmental data supported the importance of the environmental parameters we selected in defining suitable habitats. From the geographic coincidence between anemone distribution and the clusters based on environmental variables, we infer that geospatial clustering has the power to delimit ranges for marine organisms within relatively small geographical areas.

Introduction

The coastal waters of the Korean peninsula have been divided into four biogeographic regions. In summarizing research to date, Kim (1986) characterized them (Fig. 1A) on the bases of water temperature, depth, tidal amplitude, turbidity, and substratum. The Yellow Sea (YS) and East Sea (ES) are cold-water areas, seasonally influenced by the Yellow Sea Cold and Liman currents, respectively, from the north; the Korea Strait (KS) and Cheju Island (CI) are influenced by the Tsuchima current, a branch of the Kuroshio current, which brings warm waters from the tropics year round. Because the major rivers of Korea flow into the YS, vast muddy intertidal areas occur along Korea's west coast.

Whether the environmental characteristics of the defined regions significantly affect the distribution of organisms, and thereby are appropriate for defining biogeographical regions, is unknown. We therefore tested whether four coastal regions of Korea could be defined using environmental variables and whether the regions correspond to animal occurrences. Using the *k*-means clustering tools LOICZView (Maxwell & Buddemeier, 2002; Maxwell, 2003) and DISCO (Smith & Maxwell, 2003), we analyzed the distribution of six environmental parameters, including two used by Kim (1986), water temperature, and tidal amplitude. To examine whether the resulting four regions had biological significance, we overlaid distribution data for 11 species of intertidal sea anemones (Cha & Song, 2001; Song & Cha, 2002).

A hierarchical clustering of the occurrence of all 20 Korean anemone species included in Cha & Song (2001) and Song & Cha (2002) (the 11

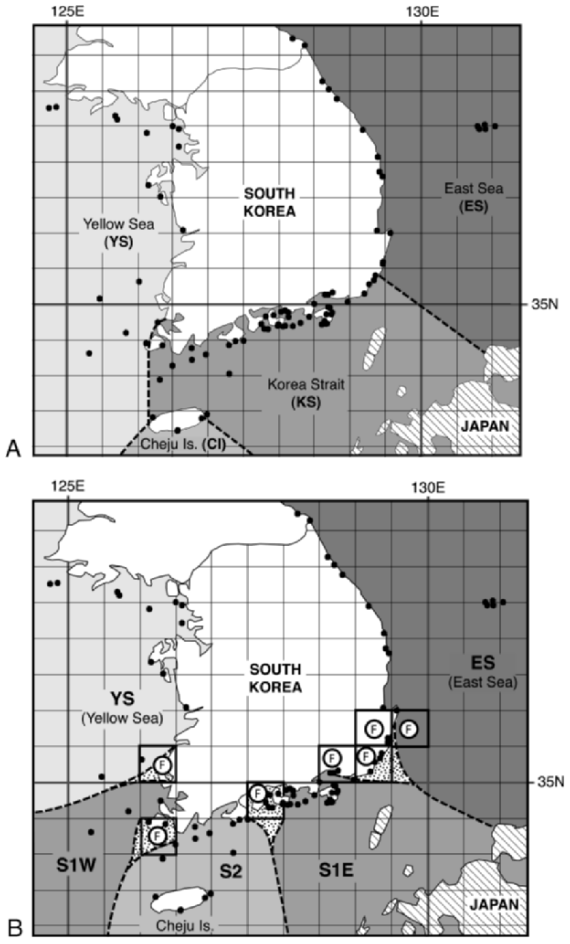

Figure 1. The southern Korean peninsula. Grid lines indicate the half-degree cells used for analysis; solid dots indicate anemone sample locations, which all fall in intertidal areas of the mainland and small islands. (A) The four coastal biogeographic regions of Korea used in analyses such as those of Kim (1986) and Song (1991). Adapted from Kim (1986) and Song (1991). (B) Biogeographic regions based on our environmental clustering and intertidal anemone distributions. Stippled areas indicate transitional zones where habitats and species characteristic of more than one region may occur. Boxed cells containing a circled F have multiple possible cluster memberships as shown by fuzzy clustering.

intertidal ones we used plus 9 subtidal ones) using Minitab resulted in four groupings (H.-R. Cha, unpublished). Thus Korean anemones appear to fall into four biogeographic regions, as Kim (1986) found for crustaceans and Song (1991) for scleractinian corals. However, in our analysis the details of the borders between them differ somewhat from those Kim (1986) postulated. The ecological significance of the pattern of clusters based on environmental parameters is supported by the distributions of the intertidal sea anemones.

Materials and methods

We used environmental data from the database 'Biogeoinformatics of Hexacorallia' (Fautin & Buddemeier, 2003); they were obtained from various sources and gridded at half-degree resolution. Continuous variables investigated included mean and maximum monthly wind speed, mean, maximum and minimum monthly sea surface temperature (SST), mean and minimum monthly salinity, annual mean strength of the spectral signal in the chlorophyll wavelength band (SEAWIFS), and tidal amplitude. The parameters were chosen because they vary through the year and are potentially important to the occurrence of intertidal sea anemones. Equal weight was given to all.

Analysis used geospatial clustering (Maxwell & Buddemeier, 2002), a technique that groups spatially distributed multi-variable data points according to the similarity of their locations in multi-dimensional space. We used the clustering tools LOICZView (Maxwell, 2003) and DISCO (Smith & Maxwell, 2003). These web-based, graphical applications were developed in support of the Land–Ocean Interactions in the Coastal Zone (LOICZ) program, and use *k*-means clustering algorithms. The tools permit clustering, classification, and comparison of environments at regional and global scales, providing a similarity analysis of the values associated with the grid cells. The two applications are fundamentally similar, but LOICZView has a function to overlay data points on clusters, while DISCO offers a fuzzy clustering capability.

To test the biological significance of the cluster results for environmental data, we plotted distributions of the 11 intertidal sea anemone species in Korea inventoried by Cha & Song (2001) and Song & Cha (2002) based on surveys done from 1968 to 2000 at 84 intertidal localities (seven additional surveyed localities are subtidal). The species (and the number of locality points for each) are *Actinia equina* (7), *Anthopleura japonica* (26), *A. kurogane* (51), *A. midori* (38), *A. pacifica* (14), *Epiactis japonica* (13), *Flosmaris mutsuensis* (3), *Haliplanella luciae* (26), *Isanthus capensis* (2), *Paracondylactis*

hertwigi (4), and *Urticina crassicornis* (5). Their localities are documented in the database 'Biogeoinformatics of Hexacorallia' (Fautin & Buddemeier, 2003). The locality data (see Fig. 1) for each species were uploaded to the LOICZView application, merged with the environmental data set, and plotted on the map using the 'merge' and 'overlay' functions of LOICZView. The same environmental data were also analyzed using the fuzzy *k*-means clustering package in DISCO, which provides a membership score for each cell in each cluster. This permits identification of localities that are intermediate between two or more clusters. In order to test the biogeographic regions proposed, we focused our attention on four-cluster tests because of Kim (1986) and because of Cha's unpublished data.

Results and discussion

Many combinations of variables yielded similar patterns; the variables we ultimately settled onto produce the results presented in Table 1, and the titles of the datasets in 'Biogeoinformatics of Hexacorallia' were tide range (scaled classes – TIDAL_RANGE), maximum windspeed (WINDSPEED_MAX), maximum SST (SST_MAX_MONTH), mean salinity (SALINITY_ANN_AVG), and the chlorophyll signal (CHLORA_SPAVGSTD). Kim (1986) also used tidal amplitude and SST in his analysis; because we dealt only with intertidal animals, we did not consider water depth or features of the sea floor, as Kim (1986) did.

Figure 1A and B contrast the borders of previously recognized areas with what we found. According to Kim (1986), three regions were initially recognized – the coasts of the East Sea (ES, the east side of the peninsula), the Korea Strait (KS, the south coast of the peninsula), and the Yellow Sea (YS, the west coast of the peninsula), including any islands lying offshore from them; a fourth region was created by separating Cheju Island (CI) from KS. The southern end of the peninsula, including Cheju Island, is primarily where our analysis (Fig. 1B) differs from previous ones: CI clusters with the south-central part of the Korean peninsula, as cluster S2 in our analysis, and our cluster S1 consists of regions in the southeastern (S1E) and southwestern (S1W) parts of the Korean peninsula and the islands off the southwest corner of the peninsula. Although cluster S1 is discontinuous, straddling S2 (Fig. 1B), S1E and S1W are more similar to each other than to any of their neighbors. YS and ES do not extend quite as far south in our analysis as in previous schemes. The environmental parameters associated with each cluster are shown in Table 1. Clusters S1 and

Table 1. Environmental parameters associated with each biogeographic region from LOICZView and DISCO clustering analysis

Environmental cluster	ES		S1	
	Value	Std Dev	Value	Std Dev
WINDSPEED_MAX	8.86	0.149	8.90	0.244
SST_MAX_MONTH	26.71	0.384	28.05	0.488
SALINITY_ANN_AVG	33.74	0.095	33.37	0.487
TIDAL_RANGE	6	0.000	1.50	1.581
CHLORA_SPAVGSTD	3.28	2.161	4.25	1.275
	S2		YS	
	Value	Std Dev	Value	Std Dev
WINDSPEED_MAX	9.48	0.253	8.89	0.222
SST_MAX_MONTH	28.17	0.312	26.79	0.759
ALINITY_ANN_AVG	32.22	0.430	32.13	0.760
TIDAL_RANGE	10	0.000	8.61	2.54
CHLORA_SPAVGSTD	3.10	1.787	3.52	1.685

S2 differ from clusters ES and YS in having higher SST, cluster S1 has the lowest tidal amplitude and the highest chlorophyll values, and S2 has the highest tidal amplitude and windspeed values.

We used the DISCO fuzzy clustering routine to identify transitional cells (typically those with less than 50% 'membership' in any one cluster). We identified seven such cells (out of a total of 47), and used their occurrence in our evaluations of the relationship between anemone occurrence and specific clusters. Fuzzy boundary zones in Figure 1B are based on the locations of cells shown by fuzzy clustering to show partial membership in more than one cluster. Although the sample size is too small for detailed analysis, anemone species distributions support our revision of Korean coastal marine biogeography.

All of the 11 sea anemone species occur also in Japan and some have much greater geographic ranges. The most widely distributed species, *Haliplanella luciae* (Fig. 2C), was recorded from all four regions (Table 2), a distribution not unexpected in this cosmopolitan species (Fautin, 2003). The other species that also occur in all four regions are *Anthopleura kurogane* and *A. midori* (Fig. 2A and B, respectively; Table 2). Overlaying occurrences of the eight species having more restricted distributions on the environmental clusters (Fig. 2, Table 2) supports the clusters as reflecting differences important to anemone distribution. Of three of the four least broadly distributed species, two are burrowers – *Flosmaris mutsuensis* (Fig. 2H) and *Paracondylactis hertwigi* (Fig. 2I). They occur in the muddy YS and at a single site on the east coast, the former at the fuzzy boundary between S1 and ES, and the latter in the northeasternmost cell of S1. Whether the rarity of the least abundant species, *Isanthus capensis*, reflects the availability of suitable habitat in only a small portion of S1 (Fig. 2K) cannot be determined with the sparse data.

Epiactis japonica (Fig. 2G) occurs mainly and *Urticina crassicornis* (Fig. 2J) occurs exclusively in ES and the fuzzy boundary between ES and S1. In the analysis of Song & Cha (2002), although *Epiactis japonica* was recorded from both ES and KS, *Urticina crassicornis* was recorded only from ES.

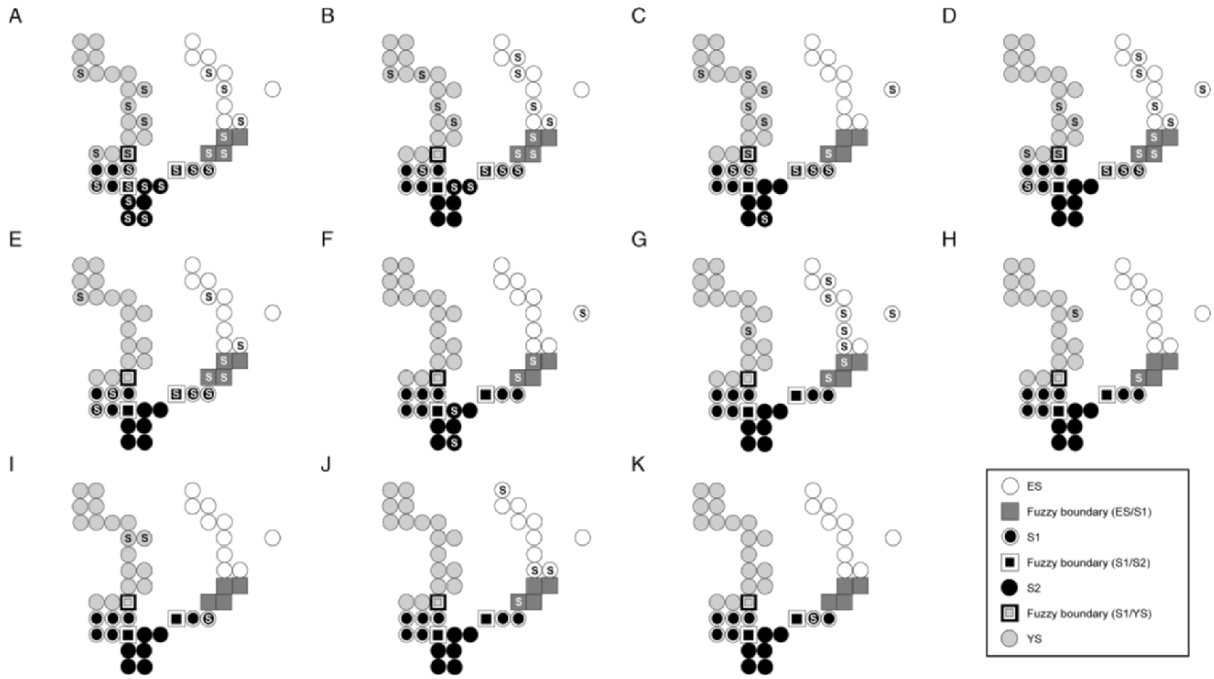

Figure 2. Species distributions of intertidal sea anemones overlain on the four biogeographic regions. The cells labeled with 'S' indicate localities at which specimens were collected or observed. (A) *Anthopleura kurogane*, (B) *Anthopleura midori*, (C) *Haliplanella luciae*, (D) *Anthopleura japonica*, (E) *Anthopleura pacifica*, (F) *Actinia equina*, (G) *Epiactis japonica*, (H) *Flosmaris mutsuensis*, (I) *Paracondylactis hertwigi*, (J) *Urticina crassicornis*, (K) *Isanthus capensis*.

Table 2. Occurrence of sea anemones by biogeographic region

Species (number of localities: map in Fig. 2)	LOICZView classification							According to Cha & Song (2001) and Song & Cha (2002)			
	ES	F (S1/ES)	S1	F (S1/YS)	YS	F (S1/S2)	S2	ES	YS	KS	CI
Anthopleura kurogane [51: A]	3	8	14	2	6	8	10	+	+	+	+
Anthopleura midori [38: B]	5	6	12		8	2	5	+	+	+	+
Haliplanella luciae [26: C]	1	2	10	1	7	3	2	+	+	+	+
Anthopleura japonica [26: D]	9	4	6	1	5	1		+	+	+	
Anthopleura pacifica [14: E]	2	4	4		2	2		+	+	+	
Actinia equina [7: F]	2	2					3	+		+	+
Epiactis japonica [13: G]	9	3			1			+	+	+	
Flosmaris mutsuensis [3: H]		1			2				+	+	
Paracondylactis hertwigi [4: I]			1		3				+	+	
Urticina crassicornis [5: J]	4	1						+			
Isanthus capensis [2: K]			2							+	

Columns labeled 'F' show occurrences with fuzzy boundaries between the two regions on either side of the F column. Distributions are plotted in Figure 2.

This difference reflects two issues: the slight shift northward in the line between ES and the region adjacent to it in our scheme relative to the previous one, and the difficulty in determining the border precisely.

In addition to YS and ES, *Anthopleura japonica* (Fig. 2D) and *A. pacifica* (Fig. 2E) occur in both disjunct parts of cluster S1, but not in intervening cluster S2 (Table 2). Their disjunct distribution supports dividing KS into two regions, S1 and S2. The environmental similarity of Cheju Island and the part of the mainland coast immediately north of it is supported by the distribution of *Actinia equina* (Fig. 2F, Table 2), which was placed by Song & Cha (2002) into ES, KS, and CI, but in our analysis occurs only in S2 and ES plus the fuzzy boundary between S1 and ES.

We conclude that the previous schemes for classifying coastal biogeographic regions in Korea were based on biologically important features, especially tidal amplitude and SST. Like Kim (1986) and Song (1991), we found good correspondence between distribution of animals and these features. Having more detailed environmental data than they and the ability to do geospatial clustering, we were able to adjust the borders of the regions more precisely; we should be able to do an even better job with more finely gridded environmental data. Despite the sparse data on species occurrence and the coarseness of the 0.5° environmental data grid, this study confirms that geospatial clustering can be applied to range data for species within relatively small geographical ranges.

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Description and ecology of *Cytaeis capitata* n. sp. (Hydrozoa, Cytaeididae) from Bunaken Marine Park (North Sulawesi, Indonesia)

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Key words: symbiosis, Gastropoda, taxonomy

Abstract

A new hydroid species, *Cytaeis capitata* n. sp. (family Cytaeididae), is described. This species, an epibiont of gastropod shells living in the shallow water of the Bunaken National Marine Park, North Sulawesi, Indonesia, is characterised by specialised polyps with an elongate tentacle, armoured by a capitation of peculiar nematocysts and lacking in the normal polyps. The medusae arise from gonophores enveloped in a layer of perisarc. Each has four oral tentacles, four radial canals, and four marginal tentacles. The diet of the polyps is related to the behaviour of gastropods: when the snails remain on the substratum, polyps are predators, whereas when the snails sink in the sediment, the polyps become sedimentivorous. The importance of the living mollusc for the hydroid is experimentally documented: the polyps living on shell fragments decrease in number by 50–60% in a couple of days and completely disappear in 8 days.

Introduction

Hydroids are especially common in shallow water rocky substrata, but they are also able to settle on living substrata like mollusc shells or bryozoans (for a review, see Gili & Hughes, 1995). One of the most common associations is that with snail shells, either living or occupied by hermit crabs (Mills, 1976; Cerrano et al., 1998). Shells are favourite substrata for hydroids of many species, and the associations range from casual partnerships to constant relationships and even to obligate symbiosis with a few or with a single species (Rees, 1967). The benefits derived from this particular association are not completely clear, but protection from predators and trophic advantages can be hypothesised, respectively, for hermit crab or snail and hydroids (Bavestrello, 1985; Brooks & Mariscal, 1985).

Many aspects of the ecology and behaviour of hydroids living in association with gastropods are known mainly from temperate areas, but in tropical seas these associations have been described quite exclusively from a taxonomic point of view.

Cerrano et al. (1998) studied the life cycle and the trophic behaviour of *Podocoryna exigua* epibiont on shells occupied by the hermit crab *Diogenes pugilator*, demonstrating the importance of the presence of the hermit crab in the shell for the life of the hydroid and the role of temperature in its seasonal cycle. Herberts (1964, 1969) studied the reproduction of *Hydractinia aculeata* living on the shells of *Nassarius pygmeus* (*Nassa pygmaea*) and the variation of the hydroid and mollusc abundance. The experiments carried out by Van Winkle et al. (2000) suggest that the hermit-crab *Pagurus longicarpus* affects the polyp number, colony morphology, and the outcome of interspecific competition of *Hydractinia symbiolongicarpus* and *Podocoryne carnea*. The symbiosis, starting at the larval stage, between *Cytaeis* sp. and the gastropod *Niotha livescens*, appears

necessary for hydroid survival, although the gastropod can survive without its partner (Inque & Kakinuma, 1992). The physiological activity of *Niotha livescens* also influences the differentiation of the medusa buds of its epibiont *Cytaeis uchidae* (Hirai & Kakinuma, 1970).

In this paper, we describe, from work done in the shallow waters of Bunaken Island (North Sulawesi, Indonesia), the new species *Cytaeis capitata,* which lives symbiotically on the shells of the nassarid *Pliarcularia globosus* and, more rarely, on the shells of an unidentified costellarid. In addition, the ecological relationships between the gastropods and their host were studied.

Materials and methods

The habitat

The North Sulawesi peninsula, which stretches between the Sulawesi Sea (NW) and the Maluku Sea (SE), lies in an area commonly considered to be the centre of marine biodiversity (Sheppard & Wells, 1988). The Bunaken archipelago, declared a Nature Reserve in 1986 and National Marine Park in 1991, is the better-known site, and is highly representative of the whole area. Shallow lagoons are found around Nain Island, along the northern coast of Montehage Island, and along the southern coast of Bunaken Island (Liang beach – Alung Banua) (Fig. 1). These lagoons are partially surrounded by mangroves, and, in the shallow water, the sandy bottom is colonised by meadows of seagrasses. The gastropods, with their epibionts, live in the soft bottom, amidst the roots of mangroves, under the seagrasses, and in the sand belt linking these two environments.

Field observations

Specimens of gastropods were sampled daily at a depth of 0–2 m in two neighbouring stations in the shallow waters of Bunaken Island. The first station was placed on the muddy bottom between the roots of the mangroves, and the second one on the sandy bottom of the seagrass bed. Twenty-five gastropods for each station were collected daily during the afternoon low tide from 15 January to 15 February 2001. For each gastropod, the following parameters were observed: species, shell life stage (young, adult), presence of the hydroid, number of polyps in the colonies, number of gonophores, distribution of the polyps and gonophores on the shell surface.

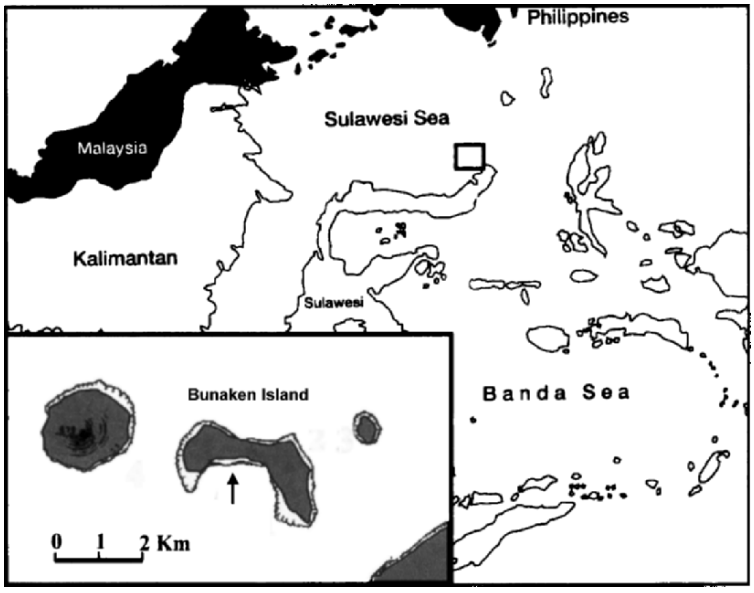

Figure 1. Map of Bunaken Island. The arrow shows the sample site.

Some shells colonised by the hydroid were used for laboratory experiments, others were fixed in formaldehyde 4% and the remains were released far away from the sampling stations, but in habitats similar to the original one.

To analyse the stomach content of the polyps, 10 shells were collected during high tide and 10 polyps, detached from the surface of each shell, were observed under the microscope.

Laboratory experiments

The effect of absence of the mollusc on the hydroid colony, both under the sand and on the surface, was studied by depriving 20 shells of the molluscs and placing 10 of them on the surface and 10 under 10 mm of sand, in a cup containing a sand layer and natural sea water, for 8 days.

To isolate a colony of *Cytaeis capitata* from the living parts of its host, the shells were broken and parts of the colonies attached to the fragments were collected to be cultured (Hirai & Kakinuma, 1973). The number of polyps and gonophores of each colony was counted daily. The colonies were fed daily with *Artemia* nauplii.

To obtain evidence of the sedimentivorous strategy of the polyps, we maintained about 50 shells, each placed in a cup with a layer of natural sand and filled with natural sea water, and we evaluated the amount of sand grains in the coelenteron of the polyps on 15 gastropods that remained for at least 2 h under the sand and on 15 that remained on the surface for the same period.

Results

Hydroid colonies were present on almost all the gastropods collected; only a few very young specimens were observed to be free of epibionts. When the shells were inhabited by hermit crabs, hydroids were always absent. About 95% of the gastropods collected belonged to the nassarid *Pliarcularia globosus,* while the remaining ones belonged to an unidentified species of family Costellaridae.

(1) Systematics

Family Cytaeididae

Cytaeis capitata n. sp.

Holotype material: Museo di Storia Naturale di Genova (MSNG) 51916, one fertile colony on shell of *Pliarcularia globosus* and several medusae, collected by S. Puce at Liang Beach, Bunaken Island, North Sulawesi, Indonesia, on 30/01/01.

Hydroid

Epizoic colony on the shell of gastropods (Figs 2 and 3a). Hydrorhiza reticulars follow grooves of host shell, protected by a thin layer of perisarc. Hydranths columnar, reaching a height of 1 mm and a width of 0.2 mm (preserved). Conical hypostome surrounded by 6–12 filiform tentacles. Tentacle nematocysts disposed in verticils; their main axis angled with respect to the axis of the tentacle, giving the tentacle a spiny appearance. A cup of perisarc at the base of each hydranth (Figs 3a and 4e). About 10 polyps per colony distributed around the shell opening (Fig. 2) characterised by one of the tentacles being longer and armoured with a capitation of large nematocysts (Figs 3a and 4a–c). Gonophores arise separately from the hydrorhiza on slender corrugated pedicels (Fig. 3a and b), developing into free medusae (Fig. 3c). Young gonophores pear-shaped, completely enveloped by perisarc (Fig. 3b). After medusa release, the empty perisarc capsule is still supported by the pedicel. The oldest gonophores are young medusae with the tentacles coiled up in the subumbrella cavity.

Nematocysts: small microbasic euryteles (7.0–7.5 × 5.0–5.5 μm, undischarged) abundant in tentacles (Fig. 4d), scattered in hydranth and hypostome; large microbasic euryteles (18–21 × 7–9 μm, undischarged) abundant in capitation of the specialised tentacle, rare and scattered in hydranth of polyps with such a tentacle (Fig. 4c and f); desmonemes (5.0–5.5 × 4.5–5.5 μm, undischarged)

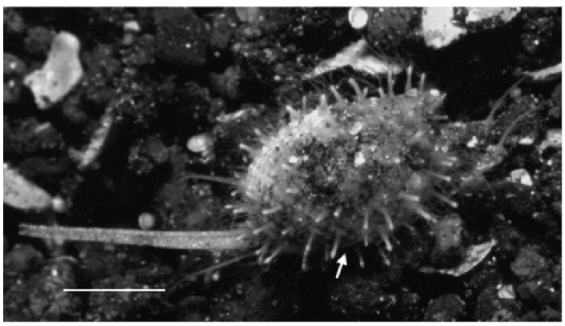

Figure 2. Photograph of *Pliarcularia globosus* with the epibiont *Cytaeis capitata.* The arrow shows the area where the polyps with the capitate tentacles are found. Bar scale: 5.0 mm.

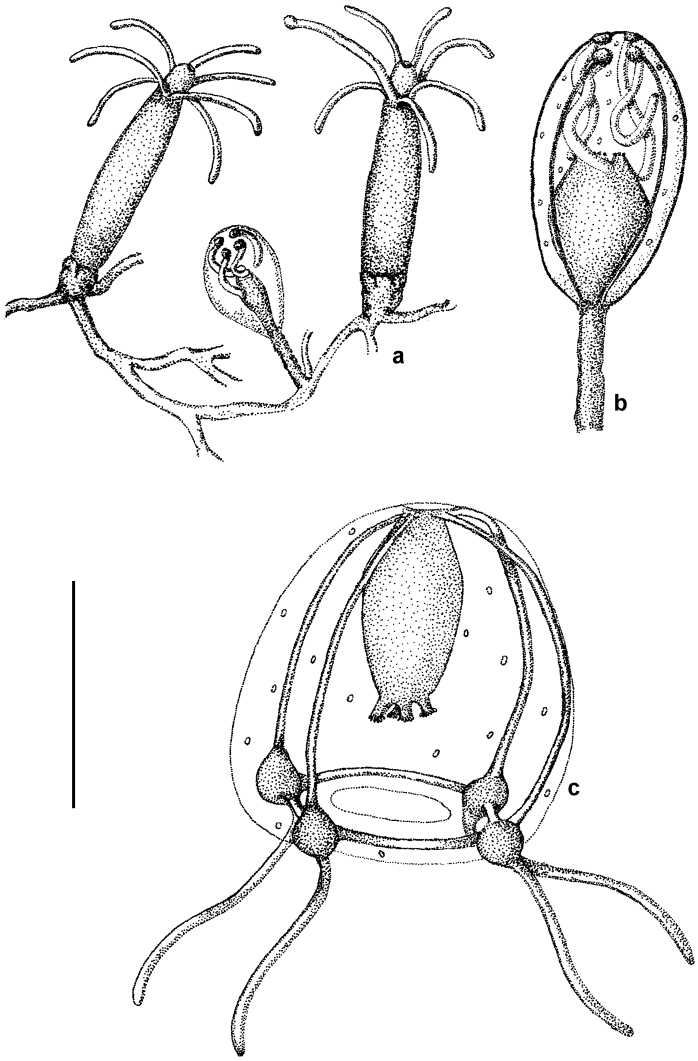

Figure 3. Cytaeis capitata. Colony showing (a) the two kinds of polyps unspecialised (left) and specialised (right) and one gonophore; (b) immature gonophore; (c) medusa. Bar scale: (a) 1 mm, (b) 0.8 mm, (c) 0.5 mm.

scattered in tentacles, in hydranth, and in hypostome.

Medusa

Just-released medusa with spherical bell 0.5 mm diameter, four radial canals, four tentacular bulbs coloured orange, four very contractile marginal tentacles (Fig. 3c). Manubrium about 2/3 of bell cavity length, pink-orange in colour, with four oral tentacles, each armoured with a terminal cluster of nematocysts. Many nematocysts scattered on exumbrella. Only immature medusae observed.

Nematocysts: small microbasic euryteles (6–7 × 5 μm, undischarged) abundant in marginal tentacles; large microbasic euryteles (10 × 5 μm, undischarged) in clusters at end of oral tentacles; desmonemes (5.0–5.5 × 4.5–5.5 μm, undischarged) abundant in tentacles and scattered on exumbrella.

Remarks

Eight species are referred to the genus *Cytaeis*, but four of these are known only from the medusa stage (Hirohito, 1988; Bouillon & Boero, 2000). The medusa of *C. capitata* is different from that of

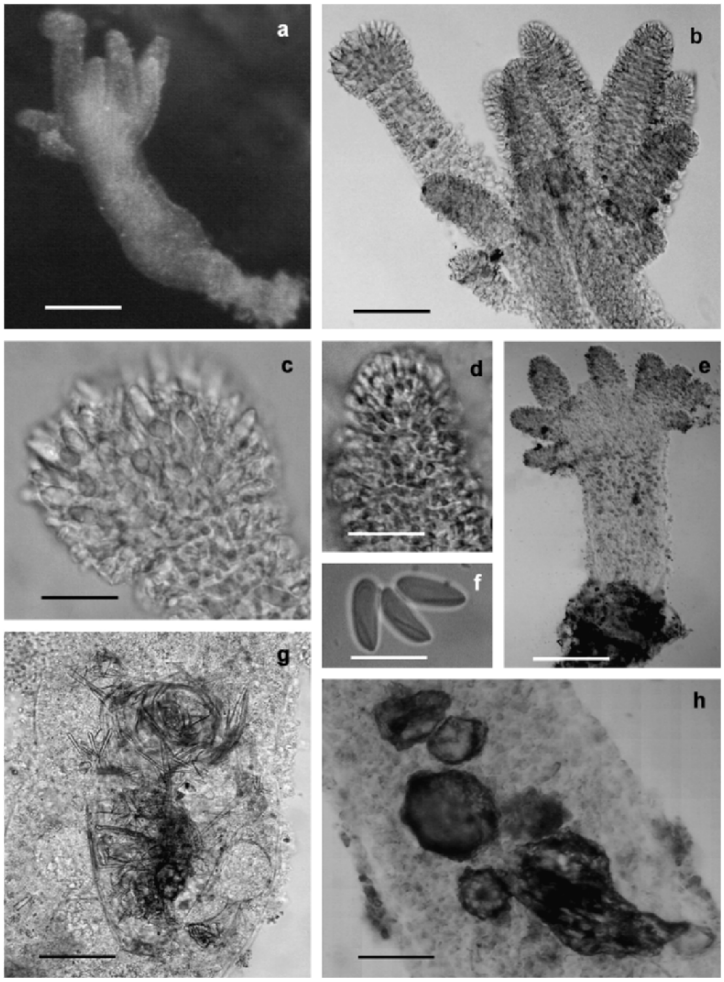

Figure 4. Cytaeis capitata. (a) Polyp with the capitate tentacle. (b) Magnification of the upper portion of the polyp with the capitate tentacle. (c) Capitation with the large nematocysts. (d) Typical tentacle. (e) Polyp with the basal cup of perisarc. (f) Large nematocysts of the capitation. (g) Stomach of the polyp containing a crustacean skeleton. (h) Stomach of the polyp containing sediment. Bar scale: (a) 0.3 mm; (b) 0.2 mm; (c, d) 60 μm; (e) 0.3 mm; (f) 25 μm; (g, h) 0.1 mm.

C. adherens Bouillon, Boero, Seghers, 1991, because of the absence of the adhesive structure at the end of the marginal tentacles, and from those of *C. pusilla* Gegenbaur, 1856, *C. tetrastyla* Eschscholtz, 1829, and *C. vulgaris* Agassiz & Mayer, 1899, because of the presence of only four oral tentacles. The adult medusa of *C. capitata* was never observed and the presence of only four tentacles is not suggestive that these might grow in number with age.

Gonophores lack the perisarc capsule, and polyps with the specialised capitate tentacle are absent in *C. nuda* Rees, 1962, *C. imperialis* Uchida, 1964, and *C. uchidae* Rees, 1962. Moreover, the polyps of the first two species lack the perisarc cup at the base.

Cytaeis nassa Millard, 1959, is very similar to the species described herein, but Millard did not report polyps with capitate tentacles.

Etymology

The name of this species refers to the presence of one capitate tentacle on the specialised polyp.

(2) Ecology and behaviour

The hydroid colonises the shells of both adult and young snails: only some very young specimens are

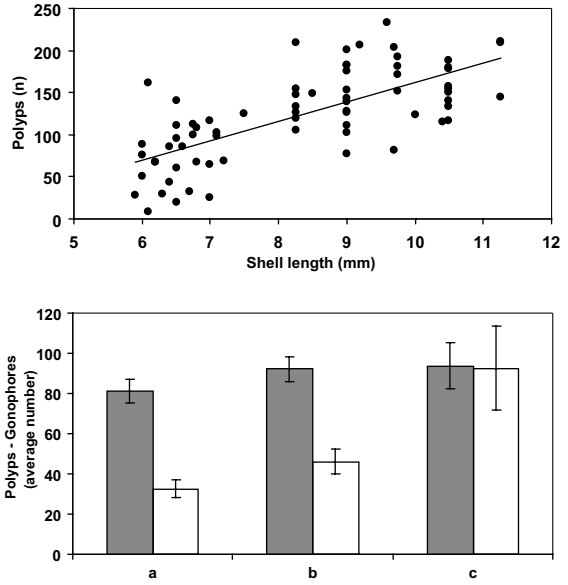

Figure 5. (upper) Size of *Cytaeis capitata* colonies (number of polyps) vs. length of host shells. (lower) Average number of polyps (grey bars) and gonophores (white bars) in colonies of *Cytaeis capitata* living on a, adult, and b, young, shells of *Pliarcularia globosus* and c, shells of Costellaridae. The differences between the number of gonophores are significant ($p < 0.05$, *t*-test).

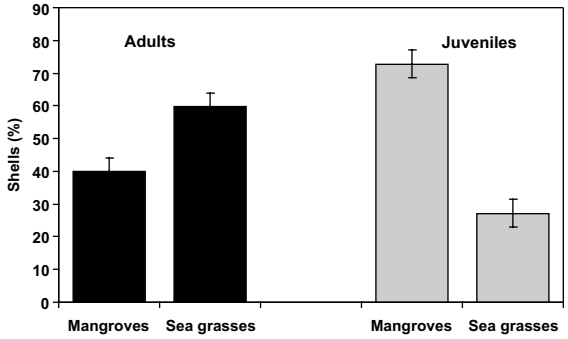

Figure 6. Spatial distribution of adult (dark grey bars) and young (light grey bars) *Pliarcularia globosus* in the mangroves and seagrass environments. Data represent the averages of 30 observations ± SE. All differences are significant ($p < 0.001$, *t*-test).

without a hydroid. The size of the colonies, in terms of number of polyps, is directly related to the length of the covered shells (Fig. 5). The average number of polyps on adult and young nassarid and costellarid shells is very similar. The presence of gonophores shows no differences between adult and young shells of *Pliarcularia globosus*, but they are more abundant in colonies living on costellarid shells (Fig. 5).

The spatial distribution of the young and adult snails is different: both live among mangrove pneumatophores and sea-grasses, but adult gastropods occupy the sea-grass environment, while the young gastropods are more abundant in mangrove sediments (Fig. 6). The mangrove habitat is subjected to long periods of emersion, when the young gastropods sink into the sediments.

When a gastropod is in the sediment, the hydranths and tentacles are very elongated, so that they may reach the surface, and, in experiments with *Artemia* nauplii, the polyps were able to capture food (Fig. 4g). On the other hand, after a long period of burrowing, the polyp coelenterons are filled with grains of sand (Fig. 4h). This sedimentivorous behaviour was also demonstrated under experimental conditions: polyps spending 2 h under the sediment have coelenterons filled with sand grains (Fig. 7). Sixty-five percent of polyps collected during high tide contain sediment in their coelenterons, and 8% have crustacean skeleton in their coelenterons.

Hydroid colonies deprived of a living mollusc show a progressive and very rapid decrease of number of polyps, regardless of whether the shells are kept on or under the sediment: after 2 days, the number of polyps decreased by 50–60%, and in 8 days, the polyps had disappeared completely. The gonophores also decreased drastically, but in colonies on the substratum surface, they showed an increase in production and release of medusae after 4 days, terminating 8 days after the culturing (Fig. 8a and b).

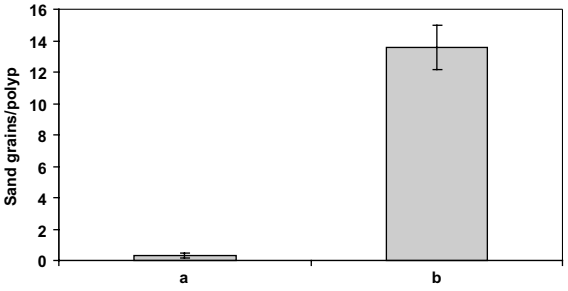

Figure 7. Average number ± SE of sand grains inside the coelenteron of polyps of *Cytaeis capitata* remained for 2 h (a) on the surface and (b) under the sediment. The differences are significant ($p < 0.001$, *t*-test).

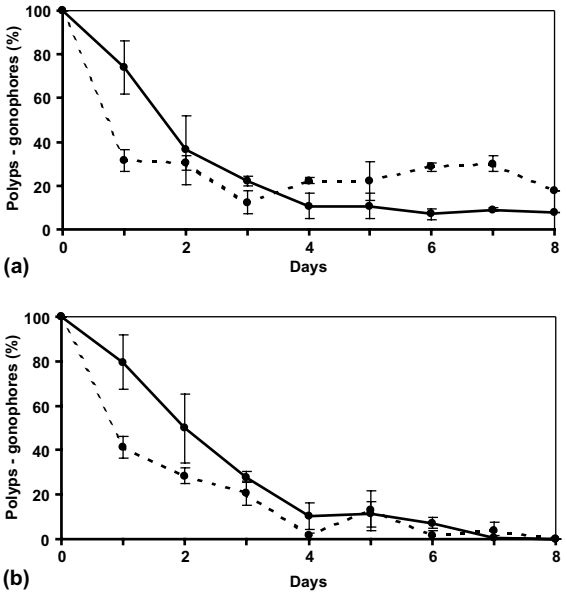

Figure 8. Number of polyps (continuous line) and gonophores (dashed line) in colonies on shell fragments placed (a) on and (b) under the sediment surface. Points represent the average from 10 tests ± SE.

Discussion

The main characteristic differentiating *Cytaeis capitata* from other known species of the genus is the presence of one capitate tentacle in some polyps that are very close to the shell opening. This tentacle appears longer than the other ones, and the capitation is armoured with very large nematocysts that are lacking in normal polyps (Figs 3a and 4a–c, f). The position of these specialised polyps (Fig. 2) is similar to the position of dactylozooids in hydractiniid hydroids living on shells, and may be interpreted as a reaction to host activity and as a defence against other organisms that live in the sand.

Edwards (1972) observed that the tentaculozooids and spiralzooids of *Podocoryna areolata* are abundant at the rim of the colony, on the outer lip of the shell host. In addition, the tentaculozooids of *Stylactaria conchicola* tend to occur along the periphery of the colony, serving some competitive function against encroaching sessile organisms, such as bryozoans, tubeworms, sponges, or other hydroids (Namikawa et al., 1992). Moreover, Buss et al. (1984) studied the hyperplastic response of *Hydractinia echinata* to allogeneic tissue, a response that shows a number of similarities to anthozoan responses to neighbours. The reaction involves the production of hyperplastic stolons and the transformation of a number of interstitial cells into nematocysts that destroy the foreign tissue.

The Cytaeididae, which belongs to the superfamily Bougainvillioidea (Petersen, 1979), is similar to Hydractiniidae, particularly in the stolonal colonies composed of naked polyps arising from a creeping hydrorhiza (Millard, 1975; Bavestrello, 1987). In addition to medusa morphology, the main difference between the Hydractiniidae and Cytaeididae definition is the polymorphism of the colony, observed in the first but absent in the second. Although Cytaedidae polyps are supposedly not polymorphic, a number of contrary pieces of evidence exist. Bavestrello (1987) described, in examining the species *Perarella propagulata* living on the gastropod *Nassarius incrassatus* (*Hinia incrassata*), some cylindrical portions of naked coenosarc surrounded by a perisarc cup. Bavestrello et al. (2000) observed in *Perarella schneideri*, an epibiont on the bryozoan *Schizoporella longirostris*, the simultaneous presence of two kinds of gastrozooid that are strongly differentiated in terms of morphology and behaviour. The filiform shape of one of these polyps, with its extensibility and shortened tentacles, suggests that it may be related to the dactylozooids of hydractiniids. Finally, we describe here a species that shows differentiated polyps that are morphologically similar to the other gastrozooids, but with a tentacle armoured like a dactylozooid and serving, in all likelihood, the same function.

The environment in which the gastropod *Pliarcularia globosus* and the associated hydroid live is characterised by two low tides each day, during which the snails assemble in pools, moving toward the open sea or sinking into the sediment. The young specimens, which live between mangrove roots, frequently sink, while adults live in the seagrass meadows. Sinking is a particularly useful strategy for the decreased salinity that frequently occurs during the wet season due to heavy rains. The lack of hydroid colonies on shells inhabited by hermit crabs is traceable to the behaviour of the crustaceans, which remain exposed to the air during low tide.

The association between *C. capitata* and its host appears to be obligate: few young shells are without hydroids, and this species was rarely found on other shells. The gastropod probably takes advantage of the protection of the hydroid's nematocysts, and the hydroids use the shell as a mobile hard substrate that permits them to exploit various feeding resources. Deeper relationships are suggested by the experiment involving the rearing of colonies deprived of the mollusc. Under these conditions the polyps completely disappear in about a week, while the gonophore production increases slightly after an initial decrease. Some hydroid colonies under stress are known to produce more gonozooids (Stebbing, 1980), and this fact indicates strong dependence of the hydroid on the mollusc. These data are similar to those reported by Hirai & Kakinuma (1973) and Cerrano et al. (1998), in which the degeneration processes occur in three and eight weeks, respectively.

The trophic strategy of this hydroid shifts from predation to sediment eating, in accordance with the behaviour of the host, which, in turn, is related to tidal frequency and salinity. Christensen (1967) reported on a predator strategy in *Hydractinia echinata* that feeds on animals belonging to the bottom fauna while the hydroid is buried in the sand. Moreover, a sediment-eating strategy was demonstrated by Cerrano et al. (1998) in *Podocoryna exigua*, a Mediterranean hydroid living on shells occupied by the hermit crab *Diogenes pugilator* and by Puce et al. (2002) in *Eudendrium racemosum*.

Acknowledgments

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Effects of anemonefish on giant sea anemones: expansion behavior, growth, and survival

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Key words: Actiniaria, symbiosis, Red Sea, predation, butterflyfish, mortality

Abstract

The symbiosis between giant sea anemones and anemonefish on coral reefs is well known, but little information exists on impacts of this interaction on the sea anemone host. On a coral reef at Eilat, northern Red Sea, individuals of the sea anemone *Entacmaea quadricolor* that possessed endemic anemonefish *Amphiprion bicinctus* expanded their tentacles significantly more frequently than did those lacking anemonefish. When anemonefish were experimentally removed, sea anemone hosts contracted partially. Within 1–4 h in most cases, individuals of the butterflyfish *Chaetodon fasciatus* arrived and attacked the sea anemones, causing them to contract completely into reef holes. Upon the experimental return of anemonefish, the anemone hosts re-expanded. The long-term growth rate and survival of the sea anemones depended on the size and number of their anemonefish. Over several years, sea anemones possessing small or no fish exhibited negative growth (shrinkage) and eventually disappeared, while those with at least one large fish survived and grew. We conclude that host sea anemones sense the presence of symbiotic anemonefish via chemical and/or mechanical cues, and react by altering their expansion behavior. Host sea anemones that lack anemonefish large enough to defend them against predation may remain contracted in reef holes, unable to feed or expose their tentacles for photosynthesis, resulting in their shrinkage and eventual death.

Introduction

The symbiosis between giant sea anemones and anemonefish is common on Indo-Pacific coral reefs, and contributes to high species diversity (Fautin & Allen, 1992). Much is known concerning the biology of anemonefishes, including the benefit they receive from the anemone host in terms of protection from predation (reviewed in Fautin, 1991; Srinivasan et al., 1999). However, little information exists about the biology of the sea anemone hosts, in terms of their sexual reproduction, growth rate, lifespan, or population turnover. Knowledge of host anemone biology is important because the stability of anemonefish populations depends in part on host dynamics.

The life history traits and morphology of giant sea anemones may be strongly influenced by their symbiosis with anemonefish. Unlike other sea anemones on coral reefs, giant sea anemones may expand large tentacles in non-cryptic habitats during daytime, presumably due to anemonefish protection of their expanded tissues against diurnal visual predators (Fautin, 1991). Anemonefish have been observed to defend the host from attack by their main predators, chaetodontid butterflyfish (Fricke, 1975; Ates, 1989; Godwin & Fautin, 1992). The anemonefish display aggressive behavior toward these predators, and experimental removal of anemonefish results in the elimination of some anemones, and the contraction of others into reef crevices (Godwin & Fautin, 1992).

Symbiotic anemonefish tend to be present in giant sea anemones (*Entacmaea quadricolor*) in warm areas but partially absent in cooler waters; the anemones appear able to survive without anemonefish in subtropical regions, possibly due to reduced predation by chaetodontid butterflyfish (Moyer, 1980). Extreme warming during the summer of 1977 enabled the appearance and breeding of reef fish in the Miyake-Jima region of Japan, and in turn possibly caused an increase in predation on giant sea anemones (discussed in Godwin & Fautin, 1992). However, it is not certain that predation caused the disappearance of these sea anemones, since changes in temperature within a short period of time also directly may cause anemone mortality.

In the Caribbean Sea, symbiotic shrimps also are known to protect host sea anemones from predation. When approached by fire worms, anemoneshrimps may exhibit aggressive behavior and push the predatory worms until they leave the shrimp's territory (Smith, 1977). Anemones without shrimps may be attacked and killed by fire worms (Smith, 1977). In addition to gaining defense against predation, anemone hosts also may benefit from anemonefish presence via nutrient transfer: ammonia excreted by the fish appears to be absorbed by the anemone and used to augment tissue growth rate and to fertilize endosymbiotic zooxanthellae (Porat & Chadwick-Furman, in press).

At Eilat, northern Red Sea, most individuals of the sea anemone *Entacmaea quadricolor* are inhabited by symbiotic anemonefish *Amphiprion bicinctus* (Fricke, 1974; Meroz & Fishelson, 1997). The ubiquitous presence of anemonefish indicates that sea anemone hosts in this subtropical reef area may depend on their fish symbionts for survival. We show here that anemonefish influence the expansion behavior, growth, and survival of giant sea anemones on subtropical reefs in the northern Red Sea.

Materials and methods

This study was conducted on patch reefs adjacent to the Interuniversity Institute for Marine Science at Eilat, northern Red Sea (29°30.11′ N, 34°55.06′ E). To examine natural variation in the behavior of sea anemone hosts with anemonefish presence, field observations were carried out at 2–4 m depth during July to August 2001 on six large (20–30 cm tentacle crown diameter when fully expanded), haphazardly selected anemones in each of three groups: those with no anemonefish, with one fish, and with at least 2 fish ($n = 18$ anemones observed total). The tentacle crown diameter of each anemone was measured 10–15× over 3–5 days. Percent expansion for each anemone was calculated as the % of observations in which the anemone was expanded to >30% of maximum tentacle diameter.

A field experiment was conducted during August to November 2001 on 10 large, haphazardly selected anemones that hosted one fish each, at a depth of 5 m. An underwater video camera was placed on a tripod 75 cm from each anemone for 9 days. The camera was connected with a cable to a monitor on the beach, to enable live viewing and continuous recording for later analysis. The field view of the camera was about 50 cm diameter, and the tentacle crown diameters of the sea anemones were 20–30 cm when fully expanded. Thus, the camera was positioned with the anemone in the center of the frame and about 10–15 cm from the anemone to the edge of the frame in all directions. Each anemone was subjected to three successive treatments, each of which lasted for about 3 days: (1) undisturbed, (2) anemonefish removed, and (3) anemonefish returned. To determine the percent expansion of each anemone (as defined above), tentacle diameter was measured every 10 min from the video frames during daylight hours.

Only individuals of the butterflyfish *Chaetodon fasciatus* were observed to prey upon sea anemones during our experiments, so the term predator will be used throughout to refer to members of this species. The following aspects of predator behavior were recorded: time after removal of anemonefish to arrival of predator, frequency of attack by predator, and duration of each attack. It was difficult to distinguish between attacks, as the camera filmed only a limited area, and it was not possible to determine whether predators remained in the vicinity between approaches to the anemone. Thus, each attack that occurred >0.5 h after a previous one was defined as a separate attack.

Night observations were made by direct observation while scuba diving (no video) on eight

of the 10 above anemones that were filmed during daytime. During nocturnal observations, we recorded the percent expansion (as defined above) of the anemones both before and after the removal of anemonefish. Each anemone was observed twice at night during each treatment.

In five of the 10 above anemones that were videotaped, we examined behavior of the anemonefish during the period before their removal. Presence of the anemonefish within 10–15 cm of the anemone, which was defined as the fish remaining in the area visible in the video frame (see above), was recorded every 10 min during the 3 days prior to their removal ($n = 6$ observations/h $\times$ 9 h/day $\times$ 3 days = 162 observations per fish).

Long-term observations on the growth and survival of *Entacmaea quadricolor* sea anemones were made annually during June 1996–2000, at 2–12 m depth. A numbered metal tag was placed adjacent to 36 large, randomly selected individuals of *E. quadricolor* (tentacle crown diameter = 20–30 cm). During each sample period, the following data were collected for each anemone: tentacle crown diameter, tentacle shape and color, column color, orientation, and number and size (total length) of associated anemonefish. Individual sea anemones were identified during subsequent surveys by their position on the reef, and individual characteristics such as tentacle shape and color, column color, orientation, and size.

Results

In field observations, the percent expansion of sea anemones varied significantly with the number of resident anemonefish (Fig. 1). Sea anemone hosts with at least two anemonefish spent a significantly higher proportion of their time expanded than did those with only one anemonefish ($X^2 = 4.45$, df = 157, $p < 0.035$) or those completely lacking anemonefish ($X^2 = 48.46$, df = 18, $p < 0.001$). Sea anemones with no fish expanded only 35.3% on average, while those with one fish were expanded more than twice as much (85.1%), and those with two fish expanded almost continuously.

During the field experiment, sea anemones that were occupied by anemonefish were about 78.2%

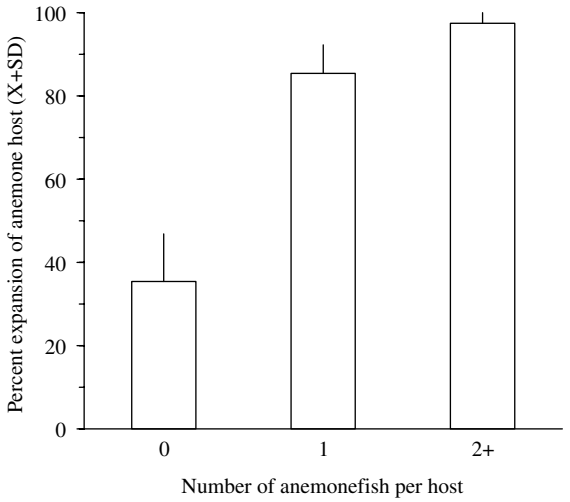

Figure 1. Natural variation in the expansion behavior of sea anemones (*Entacmaea quadricolor*) with the number of resident symbiotic anemonefish (*Amphiprion bicinctus*) in each host at Eilat, northern Red Sea. $n = 6$ anemones in each group.

expanded. Their percent expansion decreased to 49.6% on average after anemonefish were removed and to only 8.4% following attack by predators (Fig. 2). Percent expansion varied significantly among the three treatments (ANOVA of paired observations, $F_{(2,10)} = 12.00$, $p < 0.01$).

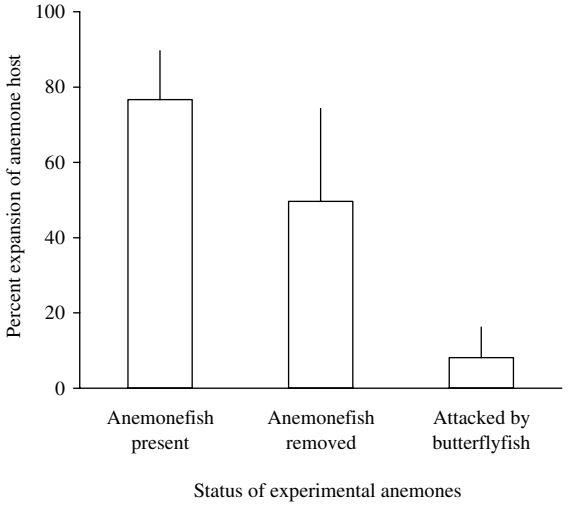

Figure 2. Experimental variation in the expansion behavior of sea anemones (*Entacmaea quadricolor*) with the presence of anemonefish (*Amphiprion bicinctus*) at Eilat, northern Red Sea. Each sea anemone was observed before and after the experimental removal of its 1 resident anemonefish, and then again after attack by the butterflyfish *Chaetodon fasciatus*. $n = 10$ anemones observed.

Table 1. Observations of predation on sea anemones (*Entacmaea quadricolor*) by butterflyfish (*Chaetodon fasciatus*) at 5 m depth on a reef at Eilat, northern Red Sea, following experimental removal of resident anemonefish (*Amphiprion bicinctus*)

Sea anemone number	Number of days observed	Time to arrival of first predators (h)	Duration of each attack (min)	Number of attacks	Number of attacks per day
1	3	3.5	3.4	5	1.7
2	2	2.7	16.1	13	6.5
3	3	2.0	0.4	4	1.3
4	3	2.0	13.3	11	3.7
5	3	1.6	1.1	15	5.0
6	2	19.0	0.6	6	3.0
7	4	45.3	5.6	6	1.5
8	3	2.0	5.4	6	2.0
9	3	0.1	1.2	3	1.0
10	3	51.3	2.5	3	1.0
X	2.9	13.0	5.0	7.2	2.6
SD	0.57	19.4	5.5	4.3	1.8
Range	2–4	0.1–51.3	0.4–16.1	3–15	1.1–6.1

During filming, predators were observed near anemones that contained anemonefish. After anemonefish were removed, predators arrived to all experimental sea anemones and preyed upon them (Table 1). The response time of butterflyfish predators varied from 6 min to almost 2 days following anemonefish removal, but in most cases they arrived within 4 h (Table 1). Butterflyfish attacked each anemone 3–15 times before the anemonefish were returned (about one to five attacks per day, Table 1). The anemones contracted immediately after attack by predators (in <1 s). In some cases, sea anemones contracted completely before predator attack, and remained contracted during the entire period that anemonefish were absent, re-expanding only after their return. For example, sea anemone #7 contracted during the entire 4 days that its anemonefish was absent (Fig. 3). All 10 anemones eventually expanded fully after the anemonefish were returned, but their time to complete expansion varied widely, from 1 h to 5 days. When returned to their hosts after about three day's absence, the anemonefish did not appear to require an acclimation period, and immediately approached the anemone host and swam among the tentacles.

During observations at night, the anemones were on average 87.5% expanded both before and after removal of anemonefish ($X^2 = 0.37$, df = 15, $p = 0.544$).

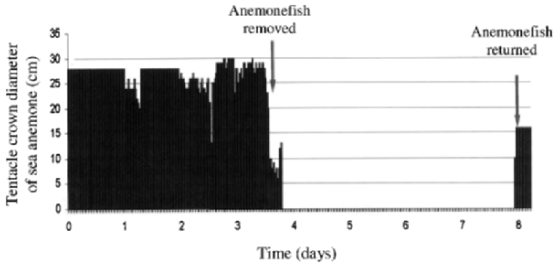

Figure 3. Example of typical behavioral response of sea anemones (*Entacmaea quadricolor*) to removal and return of resident anemonefish (*Amphiprion bicinctus*). Bars represent observations made each 10 min during daytime hours only (07:00–16:00, 6 observations/h × 9 h/day = 54 observations/day), on sea anemone #7 over 8 days.

Undisturbed anemonefish spent a significantly higher proportion of time within 10–15 cm of their anemone hosts when the host was contracted than when the host was expanded. The percent time spent within 10–15 cm of the host also varied significantly between anemonefish, from 10 to 60 s per min (=15–100% of the time, two-way ANOVA, anemonefish $F_{(1,691)} = 7.709$, $p < 0.018$; anemone state $F_{(1,4)} = 6.39$, $p < 0.005$, Fig. 4).

The behavior of anemonefish also varied with their size. When we approached the anemone host,

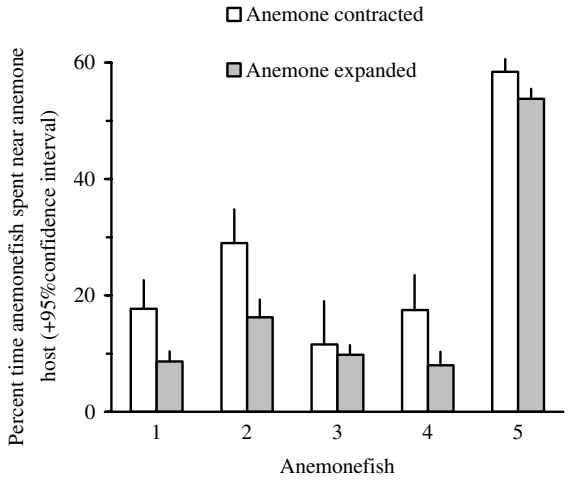

Figure 4. Variation in the behavior of five anemonefish (*Amphiprion bicinctus*) with the expansion state of sea their anemone hosts (*Entacmaea quadricolor*) at Eilat, northern Red Sea. $n = 162$ observations of each fish in each anemone expansion state. Fish were considered to be near the host if within 10 cm distance.

most of the small fish retreated and hid among the anemone tentacles, whereas the large fish approached us and exhibited aggressive behavior such as biting.

The long-term survival and growth of the sea anemones increased with the number of resident anemonefish (Fig. 5a). 84.6% of the hosts with 0.0–1.0 anemonefish ($n = 13$) did not survive over 4 years; they shrank gradually from their full size of 20–30 cm tentacle crown diameter, and eventually disappeared. In contrast, anemones with an average of 1.1–2.5 fish shrank less, and some even grew; only 4.5% ($n = 23$) died during the 4 years of observation (Fig. 5a).

Host growth and survival also depended on the total length of all fish residents. Hosts with only juvenile anemonefish (<5 cm length) mostly died, while almost all of those with adult anemonefish (>7 cm length) survived and grew (Fig. 5b).

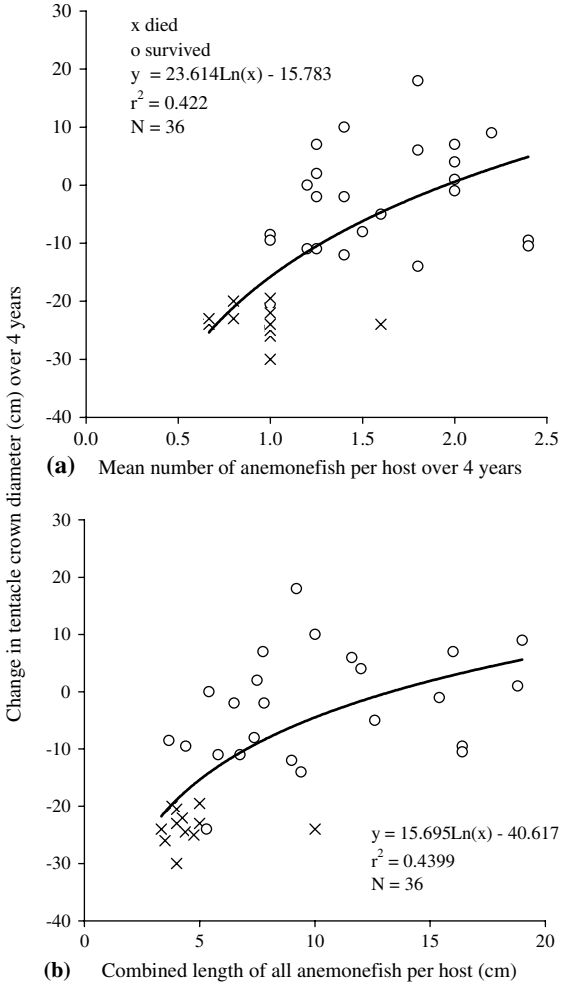
x died
o survived
y = 23.614Ln(x) - 15.783
r2 = 0.422
N = 36
Change in tentacle crown diameter (cm) over 4 years
(a) Mean number of anemonefish per host over 4 years
y = 15.695Ln(x) - 40.617
r2 = 0.4399
N = 36
(b) Combined length of all anemonefish per host (cm)

Figure 5. Long-term variation in the rates of growth and survival of sea anemones (*Entacmaea quadricolor*) with the (a) number and (b) size of resident anemonefish (*Amphiprion bicinctus*) at Eilat, northern Red Sea. Number and combined lengths of anemonefish were calculated as mean values per host from annual observations 1996–2000.

Discussion

We show here that individuals of the giant sea anemone *Entacmaea quadricolor* at Eilat have higher rates of tentacle expansion, growth, and survival in the presence of anemonefish than in their absence.

Individuals of *E. quadricolor* apparently are able to sense the presence of anemonefish, and to vary their expansion behavior accordingly (Figs 1 and 2). This is the first published report of perception of macrosymbiont presence by sea anemones. Previous observations also indicate that individuals of the sea anemone *Calliactis* sp. may detect the presence of hermit crab symbionts, and become expanded when the crabs handle them during transfer to mollusc shells (anonymous reviewer, personal observation). The type of sensory

information used by sea anemones to perceive their symbionts is not yet clear. Some anemonefishes have been shown to respond to chemical cues secreted by the anemone host (Murata et al., 1986; Arvedlund et al., 1999), but not vice versa. The behavioral response of host anemones to their fish symbionts appears to be adaptive, in that it allows them to expose their endosymbiotic zooxanthellae to light when in the presence of anemonefish, but to contract and thus defend themselves from predation when anemonefish are absent. The sea anemones possess a high level of sensitivity to anemonefish presence in that they can perceive the number of fish residing among their tentacles (Fig. 1).

We observed here that *Entacmaea quadricolor* sea anemones also respond behaviorally to attacks by their major predators at Eilat (Figs 2 and 3), as observed previously in Australia and Papua New Guinea (Godwin & Fautin, 1992). Complete contraction in response to predator attack benefits the anemone by protecting its tissues from consumption, because the predator cannot reach into the reef holes occupied by the contracted anemones. However, during contraction, the anemone cannot feed on zooplankton, absorb light for photosynthesis, or effectively absorb dissolved nutrients excreted by fish symbionts (Porat & Chadwick-Furman, in press). Thus, if the anemone host remains contracted for a long period (more than several days), it likely suffers energetically, leading to shrinkage of tissues.

The main predators of *Entacmaea quadricolor* at Eilat appear to be chaetodontid butterflyfishes, as observed at other reef localities (Fricke, 1975; Harmelin-Vivien & Bouchon-Navaro, 1981; Sano, 1989; Godwin & Fautin 1992). In the northern Red Sea, *Chaetodon fasciatus* is common in shallow waters on the reef flat and shallow slope (Bouchon-Navaro, 1980). It is also the only butterflyfish locally to specialize on sea anemones; of seven fish predators in nearby Aqaba that were examined for stomach contents, only *C. fasciatus* contained sea anemones (Harmelin-Vivien & Bouchon-Navaro, 1981).

The observed variation in response time of the butterflyfish to removal of anemonefish from their hosts at Eilat (Table 1) likely depends on where the butterflyfish is located in its territory at the time of anemonefish removal. Individuals of *Chaetodon fasciatus* guard reef territories, and they may not be within sight of a given anemone in their territory when it becomes vulnerable to predation. However, their rapid response time (13 min on average, Table 1) indicates that *C. fasciatus* usually are not far from any given anemone in their territory at this site.

The large number of repeated attacks on each anemone (Table 1) indicates that butterflyfish return repeatedly over several days to unprotected anemones. In contrast to observations on *Entacmaea quadricolor* in Australia (Godwin & Fautin, 1992), none of the individuals at Eilat were completely consumed by butterflyfish after the removal of anemonefish. All re-expanded when the anemonefish were returned, and their size and subsequent growth rates did not differ from those of anemones which had not been manipulated (N.E. Chadwick-Furman, unpublished data). It appears that a limited period of attack by predators (2–4 days), and consequent contraction of tentacles, is not enough to affect these anemones in the long-term.

The observed expansion of *Entacmaea quadricolor* anemones at night, regardless of whether they have anemonefish or not, indicates that their behavioral response to anemonefish (Figs 2 and 3) is sensitive to light. Zooxanthellate stony corals are known to alter their expansion state mainly in response to light, but also to some extent in reaction to water motion and zooplankton presence (Levy et al., 2001). It appears that *E. quadricolor* individuals expand in both light and dark conditions, but that full expansion in the light is accomplished only in the presence of anemonefish (Figs. 2 and 3). Thus, the anemones avoid contracting at night, when their main predator is inactive (Hobson, 1973).

Variation in the behavior of anemonefish with the expansion state of the host (Fig. 4) indicates that the fish also sense the condition of the host. Close proximity to the host anemone when it is contracted may benefit anemonefish, by affording them increased protection at a time when the host offers a relatively small surface area of stinging tentacles. Wide variation between anemonefish in their behavior (Fig. 4) may relate in part to fish size. Small fish are less aggressive than are large anemonefish, and remain nearer to their hosts (see Results). In addition, variation between the hosts

in their exposure to current, or in the proximity of neighboring anemones, also may influence anemonefish behavior.

The dependence of long-term growth and survival of *Entacmaea quadricolor* at Eilat on both the number and size of their resident anemonefish (Fig. 5) demonstrates strong benefits of this association to the host. Benefits provided to the host appear to be of at least two types: (1) defense from predation (Godwin and Fautin, 1992, Fig. 5), and (2) contribution of inorganic nutrients (Porat & Chadwick-Furman, in press). The size threshold for growth benefits to these large anemones (above which they begin positive growth) appears to be at least 7 cm total anemonefish length, usually in the form of one adult fish (Fig. 5). Our behavioral observations suggest that smaller fish are rarely aggressive, and are unable to effectively defend hosts from attack. In addition, they excrete less ammonia than do larger fish, and thus contribute fewer nutrients toward anemone tissue growth.

These results are the first demonstration of the importance of anemonefish to host anemones on a high-latitude, sub-tropical reef (29° N), and show that in the Red Sea, dependence on anemonefish is not reduced with latitude, as proposed for reefs in Japan (Moyer, 1980). Our observations on both short-term behavioral and long-term population processes suggest that at this site, anemones that lose their anemonefish, or who possess only small fish, may not be able to expand fully over a long period of time, due in part to repeated attacks by predators (Table 1). Contracted individuals may not be consumed immediately as occurs at other sites (Godwin and Fautin, 1992), but may eventually starve and die due to their inability to consistently feed or expose their tentacles to light.

The finely tuned behavioral responses of giant sea anemones to their symbiotic fish, and their use of nitrogen excreted by the fish, show how extensively the biology of the host anemones is influenced by this symbiosis. Further investigation will likely reveal additional mechanisms by which anemonefish contribute to their host anemones, such as possible hydromechanical reduction of the boundary layer for gas exchange around the host tentacles, due to movement by the anemonefish.

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Predation of freshwater jellyfish on *Bosmina*: the consequences for population dynamics, body size, and morphology

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Key words: *Craspedacusta*, Hydrozoa, Cladocera, morphological defense, enclosure experiment

Abstract

Invertebrate predators may cause strong changes in behaviour, life-history, and morphology of prey species. However, little is known about the influence of jellyfish on such characteristics of their prey. This study analyses the impacts of the freshwater jellyfish *Craspedacusta sowerbii* on life history and morphological defenses in a population of the cladoceran *Bosmina longirostris*. Length of mucro and antennule, size-dependent number of eggs, size at maturity, and size of juveniles, adults, and egg-carrying females were investigated during a 23 days experiment using medusae-enriched and control enclosures filled with natural plankton populations. Significant differences in parameters investigated were found not only between treatments, but also within treatments over time. Changes in *Bosmina* life-history parameters and morphology in controls were probably due to predation by cyclopoid copepods. The significant increase in the size of adults and egg-carrying females as well as the increase in mucro and antennule length in medusae-enriched enclosures are discussed as defense strategies against the freshwater jellyfish.

Introduction

Predation can have a strong impact on prey communities by influencing the evolution of defense strategies in the prey (Agrawal et al., 1999). Because in plankton communities, the environment offers few refugia for avoiding predators, prey have evolved adaptations to reduce predation risk. The presence of predators can induce life-history (Crowl & Covich, 1990), behavioural (Stich & Lampert, 1981), and morphological (Stemberger & Gilbert, 1987; Boersma et al., 1998) changes in zooplankton species. Many such traits develop only in the presence of predators. For example, selectivity for larger zooplankton and induction of diel vertical migration is well documented for fish (Brooks & Dodson, 1965; Stich & Lampert, 1981). Smaller zooplankton, in contrast, may be more vulnerable to invertebrate predators. Therefore, allocation of more energy to body growth (Chase, 1999) and lengthening of body appendages such as helmets and neckspines of *Daphnia* can be effective against invertebrate predators (Repka et al., 1995; Sell, 2000) because those like *Leptodora*, *Chaoborus*, and predatory copepods, which have to hold and manipulate prey items after capture, might be affected by bulky appendages of their prey.

In this study, the predatory influence of the freshwater jellyfish *Craspedacusta sowerbii* Lankester, 1880, on life-history and morphological traits of the small cladoceran *Bosmina longirostris* O.F. Müller, 1785, was investigated. *Bosmina* shows a variety of phenotypes, with many species exhibiting large differences in body size, shape, and length of appendages (Lieder, 1996). Field observations (Kerfoot, 1977; Sprules et al., 1984) as well as laboratory experiments (Kerfoot, 1987) on bosminids suggest that these characteristics are

influenced by the presence of predators. In lakes with high invertebrate predation pressure, small bosminids tend to have longer appendages for a given body size than they do in lakes with few predators; large, less vulnerable bosminids do not show this pattern. As shown experimentally, large omnivorous calanoid copepods can be indirectly responsible for significant increases in mucro and antennule lengths and egg size (Kerfoot, 1977, 1987). This allocation to defense may be energetically balanced by fewer eggs (Kerfoot, 1977). Additionally, juveniles tend to allocate energy to somatic growth to achieve a size refuge from predation. Recent studies demonstrated that bosminids with extreme morphological traits might be less vulnerable to predation by *Leptodora* (Hellsten et al., 1999) and small cyclopoid copepods like *Mesocyclops* (Chang & Hanazato, 2003).

Little is known about the influence of freshwater jellyfish on life-history and morphological characters of zooplankton (Purcell, 1997), although there is increasing evidence that jellyfish can significantly influence zooplankton abundance and community structure, with cascading trophic effects (Purcell, 1997). For example, in a long-term enclosure experiment, *Craspedacusta* significantly reduced densities of crustacean plankton, which indirectly boosted phytoplankton blooms (Jankowski & Ratte, 2001). During this study some evidence for differences in life-history traits and appendage lengths was obtained. Data from this enclosure experiment were re-analysed to investigate the following question: Do populations of *B. longirostris* respond to *C. sowerbii* by (1) being larger at maturity, (2) allocating more energy to somatic growth and less to egg production at smaller-sized stages, and/or (3) increasing mucro and antennule lengths?

Methods

Study site

Lake Alsdorf is a eutrophic (mean $P_{\text{total}} = 0.124$ mg l^{-1}) pond near Aachen, Germany, with a surface area of 3.1 ha, a mean depth of 2.6 m, and a maximum depth of 4.1 m. Protection from wind causes a stable thermal stratification with an anoxic hypolimnion that persists through the summer (Strauss & Ratte, 2002). Roach (*Rutilus rutilus*) dominates the fish community, accounting for 80% of fish by numbers. The total fish biomass was estimated, by electrofishing and echosounding in 1997, as ca. 350 kg ha^{-1} (Strauss & Ratte, 2002). Due to the heavy predation by roach and perch, the zooplankton is dominated by small bosminids, cyclopoid copepods, and rotifers. In 1995 and 1996, a bloom of the freshwater jellyfish *Craspedacusta sowerbii* was observed with up to 1000 individuals m^{-2}.

Enclosure experiment

A 23 days enclosure experiment was carried out from mid-June to July 1996. Six enclosures (100 μm polyethylene/polyamide sheeting, 2 m long, 1 m diameter, 1800 l) were filled with 800 μm filtered lake water to exclude medusae and fish (controls). Three of these enclosures were enriched with jellyfish at 800 individuals per enclosure, reflecting the 1995 abundance of nearly 1000 individuals m^{-2}. On each sampling day, temperature was measured, and samples were taken at 0, 1, and 2 m depth with a 'Ruttner' water sampler and mixed.

This mixed sample was subsampled to measure chlorophyll *a* and phyto- and zoo-plankton (Jankowski & Ratte, 2001). For zooplankton, three subsamples of 1 l each were filtered through a 50-μm sieve and preserved in 70% ethanol. Each subsample was counted using an inverted microscope at 50×. Life-history parameters of the experimental bosminid populations were investigated using the first 50 females of each subsample. Both body length and egg number of a total of 3199 individuals were determined. To estimate abundance of adult and juvenile females and size at maturity (SAM), the smallest egg-carrying female of each enclosure on each sampling day was determined. Females smaller than the smallest egg-carrying female were considered to be juveniles, whereas larger females were designated as adults. Differences in the time course of the investigated parameters were analysed using repeated measures analysis of variance (SAS Institute, 1988). Length of mucro and antennule were measured in around 50 individuals at the beginning and the end of the experiment for each enclosure.

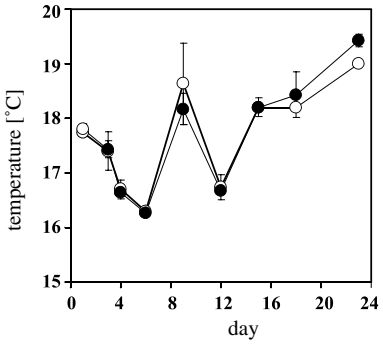

Figure 1. Time course of temperatures (mean ± SE) in medusae-enriched (black) and control (white) enclosures.

Results

Temperatures in the enclosures, ranging from 16 to 19 °C, were not different between treatments (Fig. 1). Copepod dynamics differed markedly between treatments (Fig. 2): in controls, the abundance of copepodids and adult copepods was 12–80 and 1–20 individuals l^{-1}, respectively, whereas in medusae-enriched enclosures copepodid abundances ranged between 1 and 12 individuals l^{-1}, and adult copepods never exceeds one individual l^{-1}.

At the beginning of the experiment, none of the bosminid life-history or morphological parameters under investigation showed significant differences between populations in medusae-enriched and control enclosures. Abundance of adult and juvenile females decreased slowly and continuously in the medusae treatment. In contrast, control populations were significantly more dense by the end of the experiment, as indicated by the significant day × treatment interaction for juveniles ($F_{5,20} = 4.38$, $p = 0.0075$) and significant treatment differences ($F_{1,20} = 7.83$, $p = 0.048$) for adults (Fig. 3). These differences were caused by significantly higher mortality and greater absolute egg production in control enclosures (day × treatment interaction, $F_{5,20} = 8.19$, $p = 0.0002$). Population size decreased in medusae enclosures even though egg production increased. However, egg production per female was relatively low, being no more than three. Egg production per adult female was slightly, but not significantly, higher in the medusae enclosures compared to controls.

Time course of SAM differed significantly between controls and medusae-enriched enclosures ($F_{5,20} = 38.66$, $p = 0.0034$). In control enclosures, mean SAM increased from around 0.225 mm at the beginning of the experiment to >0.275 mm, whereas in the medusae enclosures, mean SAM did not change appreciably from beginning to end of the experiment (Fig. 3). Size of egg-carrying females ($F_{5,20} = 20.42$, $p < 0.0001$), adults ($F_{5,20} = 12.20$, $p < 0.0001$), and juveniles ($F_{5,20} = 4.40$, $p < 0.0073$) all varied significantly during the experiment and were larger in controls (Fig. 3). Whereas juveniles stayed around 0.2 mm in the medusae enclosures, they grew to 0.25 mm in controls. The average size of adult females and egg-carrying females increased in control and medusae enclosures, but effects were more pronounced in controls. The mean size of egg-carrying females rose 40% in controls but only 20% in medusae enclosures. On average, adult females increased about 0.1 mm in controls and around 0.025 mm in medusae treatments.

Size-dependent egg production differed strongly between medusae-enriched and control

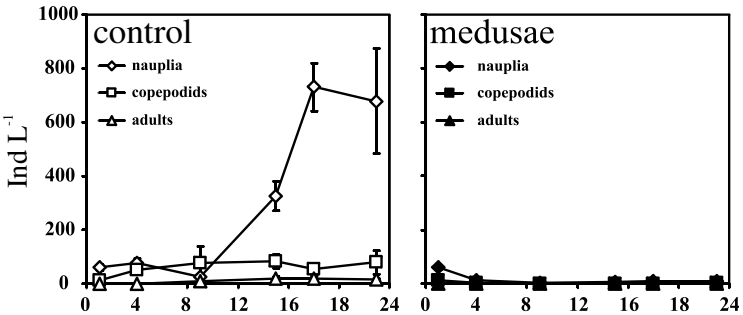

Figure 2. Time course of abundance dynamics of copepod stages (mean ± SE) in medusae-enriched (right) and control (left) enclosures. Ind, number of individuals.

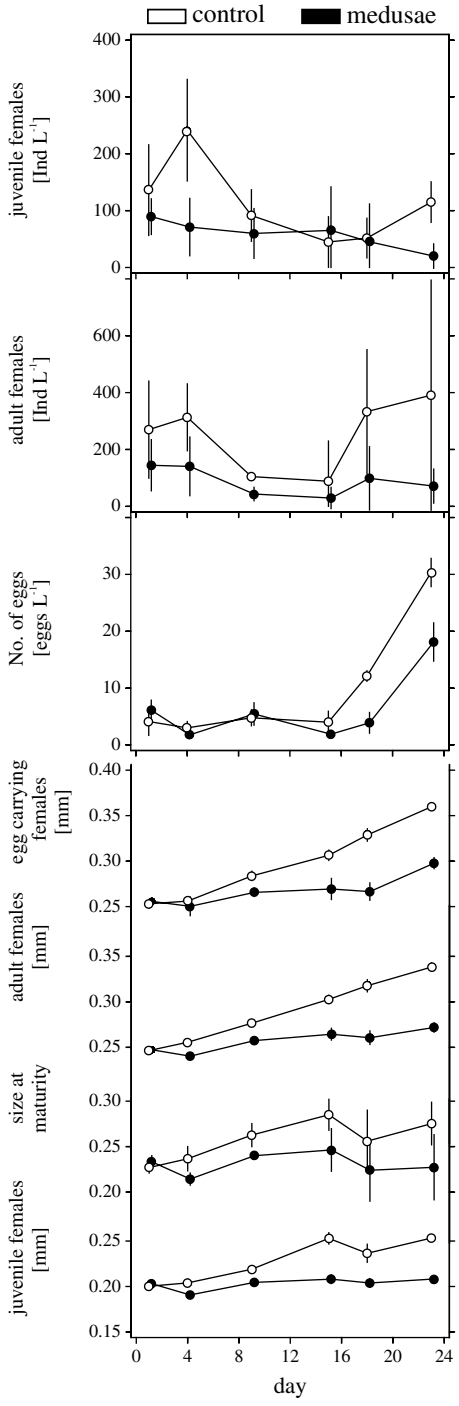

Figure 3. Time course of the population dynamics, life-history characteristics, and body size of several stages of the *B. longirostris* population in medusae-enriched (black) and control (white) enclosures. Shown are mean ± SE of three replicates. From top: abundance of juvenile females, abundance of adult females, abundance of parthenogenetic eggs, size of egg-carrying females, size of adult females, size at maturity, and size of juvenile females. Ind, number of individuals.

enclosures (Fig. 4). In particular, small adult females, between 0.25 and 0.35 mm, produced significantly more eggs in medusae enclosures than in controls ($F_{1,55} = 19.31$, $p = < 0.0001$), including body size as a co-variable. Bosminids in medusae enclosures had not only a smaller SAM but also produced more eggs.

At the beginning of the experiment there were no differences in mucro or antennule length between medusae and control enclosures (Figs 5–7). Initial mucro length, which ranged from 2 to 28 μm, increased over the experiment in both treatments to <56 μm. When body size is included as a co-variable, mucro length increased significantly in both treatments over time and was significantly higher in controls ($F_{3,172} = 14.68$, $p < 0.0001$). Additionally, body size ($F_{1,172} = 88.96$, $p < 0.0001$) and body size × treatment interaction ($F_{3,172} = 6.90$, $p = 0.0002$) had a significant effect on mucro length, the increase in mucro length being greater in large, adult females in control enclosures (156%, Fig. 7) than in medusae enclosures, in which it also increased (66%). Juvenile females showed a different pattern: significant differences were found only in the controls at the end of the study, with an increase from 13.0 to 29.9 μm (130%). Nearly the same pattern was observed for antennule length (Figs 6 and 7), but the lengthening was not as strong as for the mucro: it increased from 43 to 91 μm at the beginning to up to 163 μm at the end. Antennule lengths were not significantly different between medusae and control enclosures at the start, but

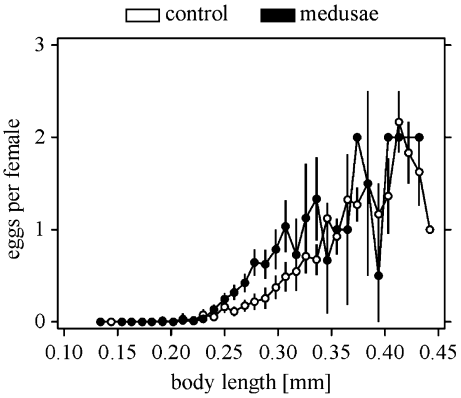

Figure 4. Differences in the average number of eggs per female of *B. longirostris* in relation to body length in medusae (black) and control (white) enclosures (mean ± SE).

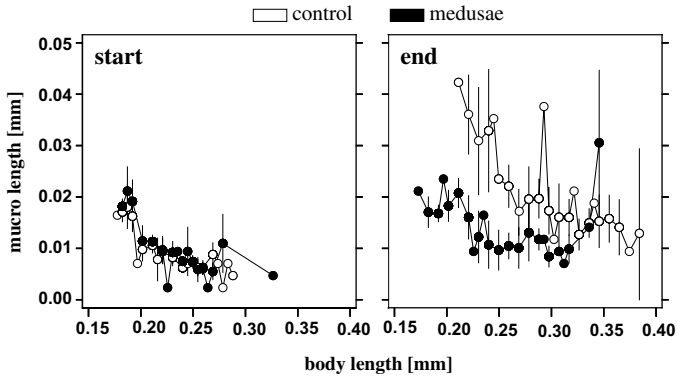

Figure 5. Differences in mucro length in relation to body length of the *B. longirostris* populations in medusae (black) and control (white) treatments (mean ± SE) at the beginning (left) and end (right) of the experiment.

were significantly longer in controls at the end. Moreover, values of antennule length for all enclosures were significantly larger at the end of the study ($F_{3,176} = 265.81$, $p < 0.0001$, Fig. 7). Antennule length of adult females increased around 79% in controls and ~14% in medusae enclosures, respectively. Juveniles showed no increase in antennule length within medusae enclosures, but increased significantly in controls (from 65 to 134 μm, 106%).

Discussion

In the enclosure experiment, there were not only significant differences in bosminid population dynamics, but also in life-history and morphological parameters. Bosminid populations in medusae enclosures showed a significantly smaller SAM and, consequently, a smaller size of juveniles, adults, and egg-carrying females. Additionally, the smaller adults produced more eggs than similar sized adults in controls. In medusae as well as control enclosures, adults developed longer mucros and antennules during the experiment, although it was more pronounced in the controls. For juveniles, a significant increase in mucro and antennule lengths was observed only in controls.

As reported elsewhere (Jankowski & Ratte, 2001), predation by *Craspedacusta sowerbii* caused significantly lower abundances of bosminids and cyclopoid copepods compared to control enclosures; indeed, adult cyclopoid copepods were absent in the medusae treatment. Cyclopoid copepods in medusae-free enclosures were dominated by small species, e.g. *Mesocyclops* sp. and

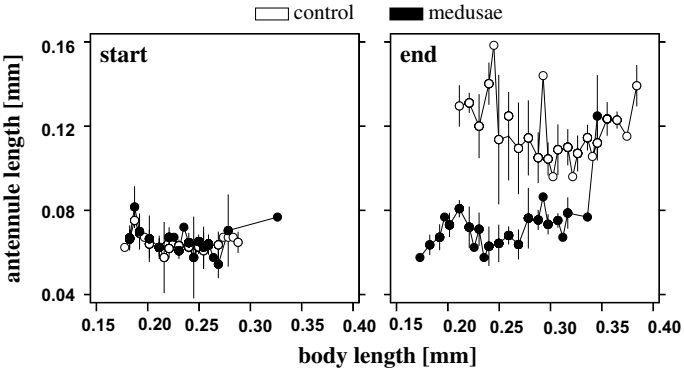

Figure 6. Differences in antennule length in relation to body length of the *B. longirostris* populations in medusae (black) and control (white) treatments (mean ± SE) at the beginning (left) and end (right) of the experiment.

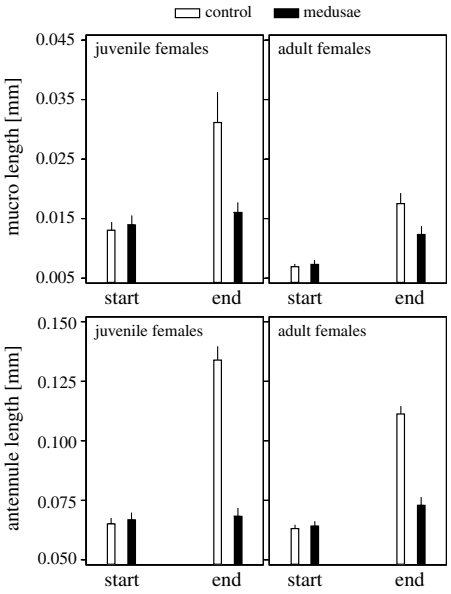

Figure 7. Mean ± SE of mucro (top) and antennule (bottom) length of juvenile (left) and adult (right) bosminids at the beginning and end of the experiment.

Thermocyclops sp. Larger species, e.g. *Marcocyclops* sp. and *Cyclops* sp., also occurred but at very low abundances. Additionally, *Craspedacusta* caused cascading effects: the lower grazing pressure by bosminids caused significantly higher chlorophyll-*a* concentrations in the medusae-enriched enclosures.

In control enclosures, where copepods reached high abundances, *Bosmina longirostris* reacted typically to predation by copepods (Kerfoot, 1987; Kappes & Sinsch, 2002; Chang & Hanazato, 2003): the population showed an increase in body size as well as mucro and antennule length. Additionally, this study strongly suggests that (1) the size at maturity increased, and (2) more energy was allocated to somatic growth than to early reproduction, thereby achieving a size refuge from predation. Hence, these differences between control and medusae enclosures were probably not caused directly by the presence or absence of *Craspedacusta*, but instead were the indirect result of predation by these jellyfish on copepods. The fact that small cyclopoid copepods, e.g., *Mesocyclops* sp., can influence the *B. longirostris* population is a relatively new idea (Kappes & Sinsch, 2002; Chang & Hanazato, 2003), because these small predators are primarily known as rotifer predators (Gilbert & Williamson, 1978; Williamson, 1980, 1983, 1984). In contrast, large copepods, like *Epischura* sp. and *Cyclops* sp., are already known to cause life-history and morphological changes in small cladoceran species (Kerfoot, 1980, 1987). Life-history plasticity of prey organisms can strongly influence the outcome of food web interactions (Chase, 1999).

In medusae enclosures there was nearly no change in size at maturity (and hence the size of juveniles), suggesting that *Craspedacusta* did not influence the life history of *B. longirostris*. This is supported by the relatively high number of eggs carried by small females. These two facts in combination suggest that these cladocerans have no need to invest in higher somatic growth to reach a size refuge, where predation risk is lower. In contrast, the size differences of adult and egg-carrying females between treatments suggests that *C. sowerbii* showed size-selective predation, jellyfish selecting larger individuals of bosminids. This is in agreement with other studies showing that prey capture increases with prey size (reviewed in Purcell, 1997), as long as escape velocities of prey are not the limiting factor (as in adult copepods). However, size selection of *C. sowerbii* seems not to be as strong as size selection by fish (Brooks & Dodson, 1965). As the enclosures were filled with natural plankton and the predation pressure by roach is pronounced in this lake (Strauss & Ratte, 2002), the increase in body size of adult females was probably caused by the absence of fish predation in the enclosures.

Although elongation of mucro and antennule during the experiment was much greater in the controls, a significant increase was also observed in the medusae treatment. In contrast to the controls, this elongation was observed only for larger females. Morphological defense appendages are costly and investment in them reduces fecundity (Kerfoot, 1977), probably because extreme morphotypes need more energy for swimming (Lagergren et al., 1997). Hence, investment in defensive appendages in the medusae treatment was not coincidental. It is surprising that increased appendage length was observed in larger females: studies of other invertebrate predators (e.g., copepods, *Chaoborus* sp., and *Leptodora* sp.) have shown predation risk to be more pronounced for juveniles, so prey juveniles often show a stronger

response to these predators than do adults (Kerfoot, 1987). Adult copepods and other invertebrate predators except *C. sowerbii* were absent in the medusae enclosures, and densities of copepodids, which feed on phytoplankton and rotifers (Gilbert & Williamson, 1978; Williamson, 1980, 1983, 1984; Hansen & Santer, 1995), were <2 individuals l^{-1} except at the start of the experiment. Hence, predators other than *C. sowerbii* could not be the reason for the observed induction of defense appendages in bosminids. In addition to predators, abiotic factors can trigger modifications in cladoceran morphology (Hanazato & Dodson, 1995; Kappes & Sinsch, 2002), but abiotic conditions such as temperature were nearly constant during the experiment (Jankowski, unpublished data).

What caused the large bosminids to invest in costly appendages? The most likely explanation is the presence of *C. sowerbii*. While there is evidence that elongation of appendages in bosminids increases handling time of them by predators and opportunities for their escape if caught by predators like *Leptodora* (Hellsten et al., 1999), this does not appear to apply to predation by *Craspedacusta*, which has a different hunting behaviour. *Craspedacusta* floats while feeding from the surface to deeper parts. During this time, the tentacles are exposed like a filter; thus water flow brings prey toward the tentacles. In marine medusae, prey species vulnerability varies with the predator's nematocyst types (Purcell & Mills, 1988) and the number and spacing of tentacles (Purcell, 1997). In consideration of this hunting behaviour, there seems to be no need for adult bosminids to produce costly appendages to reduce the handling time for medusae.

In conclusion, this study showed that *Craspedacusta* induced no direct life-history changes in *B. longirostris*, but induced morphological defenses like lengthening mucro and antennule. Further investigation is needed to resolve the mechanisms that were involved.

Acknowledgments

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Associations between gelatinous zooplankton and hyperiid amphipods (Crustacea: Peracarida) in the Gulf of California

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Key words: symbiosis, plankton, siphonophores, medusae, ctenophores

Abstract

Hyperiid amphipods are pelagic crustaceans that live associated with gelatinous zooplankton including medusae, ctenophores, siphonophores, and salps. Standard plankton sampling disrupts natural associations, so the most reliable way to determine an association is through direct observation of the organisms in their environment. The planktonic fauna of the Gulf of California dwelling between 10 and 3000 m was surveyed using SCUBA diving and a remotely operated submersible (ROV) during March 2003. Here we report our observations on a total of 14 symbiotic associations found between the hyperiid amphipods and various taxa of gelatinous zooplankton. We found parental care behavior in a group of amphipods (Oxycephalidae) in which this phenomenon has not been previously reported. For two hyperiid species, *Euthamneus rostratus* and *Vibilia australis*, we present the first information on their symbiotic relations. Additional hosts were discovered for other well-known and widely distributed hyperiid species (i.e. *Brachyscelus crusculum*, *Hyperoche medusarum*). Photographic evidence of some of these interactions is included in this contribution. This is the first survey of these relationships in the Gulf of California, and many aspects of the ecology and biology of these symbioses remain to be studied.

Introduction

Hyperiid amphipods have been advanced as a polyphyletic group (Bowman & Gruner, 1973), bringing together the descendants of different lineages of benthic forms that have developed a benthic-like existence on the pelagic substratum provided by gelatinous zooplankton (Laval, 1980). The relatively low host specificity and the morphological evidence provided by the analysis of the mouthparts suggest that parasitism in hyperiid amphipods has evolved only recently (Dittrich, 1988, 1992). Hyperiids are associated with different kinds of gelatinous zooplankton at the onset of their existence, when they are assumed to be strict parasites (Dittrich, 1987, 1992); the duration of the association depends on the hyperiid species and varies according to biological and ecological factors. The relationship is nearly always detrimental to the host, although Vader (1983) categorized the different types of hyperiid relations as ectocommensalism, endocommensalism, protection, and micropredation, while hyperiids also obtain buoyancy and transportation. It is assumed that most hyperiid amphipods are not biologically suited to a pelagic free-living existence (Laval, 1980).

Some genera and even families appear to be restricted to associations with certain host groups, but we are far from being able to know the mechanisms or specificity for host selection. The juveniles expelled from the pouch, in many cases

without swimming appendages (Dittrich, 1992), would have a very little likelihood of encountering a host by themselves. The adult female does not produce a large number of eggs to compensate for this low probability of host encounter; therefore, she is responsible for the dissemination of her limited progeny, seeking the correct hosts and demarsupiating on them.

Prior works have described some of these peculiar symbiotic interactions (Madin & Harbison, 1977; Thurston, 1977; Laval, 1980). In this contribution, we report our observations on the interaction of hyperiids with different gelatinous zooplankton obtained during surveys of the water column between the surface and 3000 m in four different areas of the Gulf of California.

Methods

The planktonic fauna of the Gulf of California was surveyed during an oceanographic cruise carried out aboard the R/V *Western Flyer* of the Monterey Bay Aquarium Research Institute (MBARI). The cruise took place between March 12 and 31, 2003, and included sampling stations in Guaymas, Pescadero, and Farallon Basins and Alarcón seamount of the southern Gulf of California (Fig. 1). We used blue-water SCUBA diving to survey the upper 20 m, and a remotely operated submersible (ROV) to sample the zooplankton fauna between 200 and 3000 m depths. Specimens were captured together with their associated hyperiids in glass jars on SCUBA dives, or using 'detritus samplers' described by Youngbluth (1984) for the ROV samples. In short, the samplers are acrylic cylinders with hydraulically actuated lids on the top and bottom. These slide closed to seal the vessel when the animal has been maneuvered into the chamber. Once brought on board the ship, the host species and its associated copepods and amphiods were examined and identified. After this initial manipulation *in vivo*, the specimens were fixed in 4% formaldehyde and preserved in a solution of propylene glycol (4.5%), propylene phenoxetol (0.5%), and sea water (95%) for further taxonomic examination. The identification of the hyperiid amphipods was made using the keys, illustrations, and descriptions of Zeidler (1992, 1998), Shih & Chen (1995), and Vinogradov et al. (1996). The hydromedusa described herein as *Aequorea coerulescens* was called *A. macrodactyla*

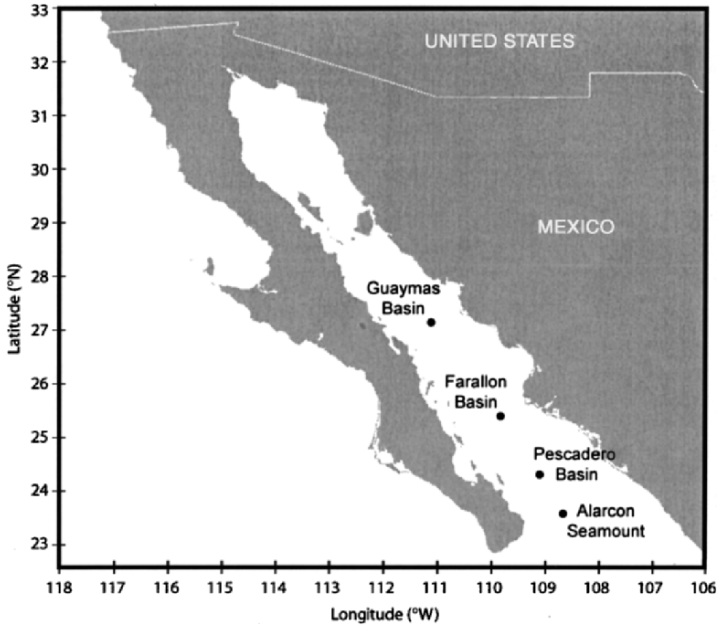

Figure 1. The Gulf of California region showing the stations where ROV and blue-water SCUBA dives were conducted.

by Stretch & King (1980) during their study of the region.

Results and discussion

We identified the following gelatinous organisms carrying hyperiid amphipods: two species of hydromedusae, and one each of scyphomedusa, siphonophore, salp, heteropod mollusc, and ctenophore (Fig. 2). Up to six species of hyperiids were recorded in association with these animals (Table 1). Previous faunistic work in the Gulf (Siegel-Causey, 1982; Brinton et al., 1986) has reported on the composition and distribution of the hyperiid fauna, but not in relation with their hosts.

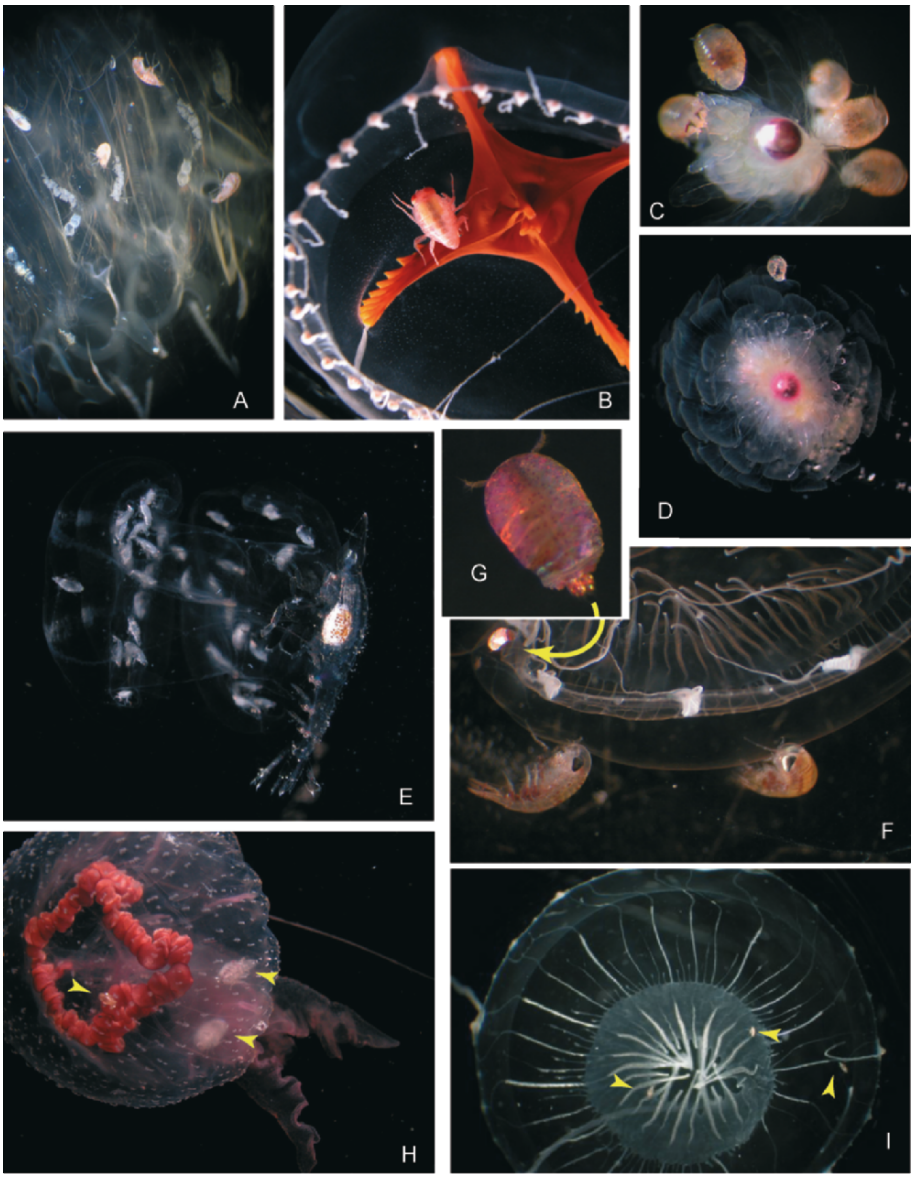

Figure 2. Amphipods and their gelatinous hosts. (A) *Vibilia australis* on *Cyclosalpa bakeri*. (B) *Hyperoche medusarum* on *Chromatonema erythrogonon*. (C, D) *Parascelus typhoides* on *Athorybia rosacea*. (E) *Oxycephalus clausi* female and juveniles on *Ocyropsis crystallina crystallina*. (F) *Brachyscelus crusculum* and (G) *Sapphirina nigromaculata* on *Aequorea coerulescens*. (H) *Euthamneus rostratus* on *Pelagia noctiluca*. (I) *Euthamneus rostratus* juveniles on *Aequorea coerulescens*.

Table 1. Amphipods (A) and copepods (Co) associated with different gelatinous taxa (M = medusae; S = siphonophores; Ct = ctenophores; H = heteropod) collected in the Gulf of California at different depths (in m) during the survey period

Associated crustacean	Host	Locality	Depth (m)	Dive
Hyperoche medusarum (Kröyer, 1838) (A) w/eggs	*Chromatonema erythrogonon* (Bigelow, 1909) (M)	Carmen Basin	1100	ROV 546
Vibilia australis Stebbing, 1888 (A)	*Cyclosalpa bakeri* Ritter, 1905 (Salp)	Alarcón Seamount	244	ROV 547
Brachyscelus crusculum (A) w/larvae in marsupium	*Pterotrachea hippocampus* Philippi, 1836 (H)	Pescadero Basin	15	BW9
Brachyscelus crusculum Bate, 1861 (A)	*Aequorea coerulescens* (Brandt, 1838) (M)	Isla Cerralvo	10	BW1
Brachyscelus crusculum (A) with eggs	*Aequorea coerulescens* (M)	Farallon Basin	15	BW3
Brachyscelus crusculum (A) & *Sapphirina nigromaculata* Claus, 1863 (Co)	*Aequorea coerulescens* (M)	Guaymas Basin	10	BW4
Brachyscelus crusculum juv. male (A) & *Sapphirina nigromaculata* (Co)	*Aequorea coerulescens* (M)	Alarcón Seamount	10	BW10
Brachyscelid (A) unidentifiable juv.	*Aequorea coerulescens* (M)	Pescadero Basin	10	BW8
Euthamneus rostratus (A) adult and juv.	*Pelagia noctiluca* (Forskål, 1775)(M)	Pescadero Basin	10	BW8
Euthamneus rostratus (Bovallius, 1887) (A) juv.	*Aequorea coerulescens* (M)	Farallon Basin	10	BW3
Oxycephalus clausi Bovallius, 1887 (A) and juv.	*Ocyropsis crystallina crystallina* (Rang, 1826) (Ct)	Pescadero Basin	10	BW2
Oxycephalus clausi Bovallius, 1887 (A) and juvs.	*Ocyropsis crystallina crystallina* (Ct)	Farallon Basin	10	BW5
Parascelus typhoides Claus, 1879 (A)	*Athorybia rosacea* (Forskål, 1775) (S)	Isla Cerralvo	10	BW1
Parascelus typhoides (A)	*Athorybia rosacea* (S)	Pescadero Basin	10	BW8

Parental care

Many benthic amphipods have shown different degrees of parental care (Thiel, 1997; Thiel et al., 1997), whereas this phenomenon seems to be quite unusual (or unknown) in the planktonic forms. Members of the hyperiid family Phronimidae are the only hyperiid crustaceans for which maternal care has been reported (Thiel, 1976, 2000). In this family, the mother feeds and keep the larvae within barrel-shaped salp tests after demarsupiation. In our study, a female of *Oxycephalus clausi*, a member of the family Oxycephalidae, was observed taking care of the juveniles demarsupiated into the ctenophore *Ocyropsis crystallina*; the female was keeping the young individuals (ca. 30 juveniles) on the surface of the ctenophore, where they were randomly distributed across the host. The female, using her pereopods, kept the juvenile-carrying ctenophore in constant motion and swam about in different directions without losing contact with the ctenophore (Fig. 2E). This behavior persisted for several hours after the time of collection. This is the first report of maternal care in this hyperiid family; further studies could elucidate if, as reported for the Phronimidae, the oxycephaliids also feed their larvae or if they feed directly on the ctenophore tissues. Demarsupiation is yet another interesting process by which the females expells the larvae from her pouch to the host surface where they can feed upon the host tissues (Laval, 1980). This phenomenon was not observed in the specimens examined herein.

Remarks on the species

Shih & Chen (1995) stated that most of the associations reported in the literature involve amphipods of the families of the Infraorder Physocephalata (except for some *Scina*); all the species recorded herein belong to this taxon.

Euthamneus rostratus is a relatively rare species, but it is widely distributed; it has been known to occur in tropical as well as temperate areas. It was found at a depth of 10 m associated with the scyphomedusa *Pelagia noctiluca* (Fig. 2H) and the hydromedusa *Aequorea coerulescens* (Fig. 2I), both of which are known to host a wide variety of hyperiids (Laval, 1980). However, there are no previous records of symbiosis of this hyperiid.

Vibilia australis is a species widely distributed in the surface waters of the tropical latitudes of the oceans. This genus is said to be restricted to salps (see Madin & Harbison, 1977), and in our case it was recovered from *Cyclosalpa bakeri* collected with the ROV. However, as noted for *E. rostratus*, this is the first information about the symbiotic relation of this hyperiid species.

Parascelus typhoides dwells in the upper 200 m layer. It is a relatively rare species distributed in tropical and temperate regions. The females of this species were recently synonymized under *P. edwardsi* Claus, 1879, by Zeidler (1998). This species has been found in the siphonophore *Forskalia ?edwardsi* Kölliker, 1853 (Laval, 1980), and in our surveys two females of this species were recorded at a depth of 10 m in association with another siphonophore, *Athorybia rosacea* (Fig. 2C and D).

Brachyscelus crusculum is a common epipelagic form, widely distributed in the oceans. This species has been found in symbiosis with several groups of pelagic organisms, mainly with salps (*Salpa fusiformis* Cuvier, 1804, *Salpa maxima* Forskål, 1775, *Thalia democratica* (Forskål, 1775)) but also with medusae (*Aequorea* spp.) and heteropod molluscs (*Pterotrachea* spp.) (see Laval, 1980). On several occasions, we found this species associated with the hydromedusa *Aequorea coerulescens* (Fig. 2F) and with the heteropod *Pterotrachea hippocampus*. The only male specimen in any of our samples was a juvenile of this species associated with *A. coerulescens*.

Hyperoche medusarum is considered to be a bipolar form; it was recorded at 1100 m depth in the Gulf of California. This amphipod has been found in symbiosis with a wide variety of pelagic coelenterates, including six species of medusae, and six ctenophores (see Laval, 1980). The finding of this species with the medusa *Chromatonema erythrogonon* has not been reported before and adds this species to the host list of *H. medusarum* (Fig. 2B). (The specimens referred to herein as *H. medusarum* show some morphological differences from previous descriptions. Hence, if the taxonomic status of these specimens changes, this record could reflect to a new association.)

Oxycephalus clausi (Fig. 2E) has been found more frequently in ctenophores such as *Eurhamphaea vexilligera* Gegenbaur, 1856, *Mnemiopsis mccradyi* Mayer, 1900, *Bolinopsis vitrea* (L. Agassiz,

1860), and *Ocyropsis maculata* (Rang, 1826), among others (Harbison et al., 1978); the list includes also *Ocyropsis crystallina crystallina* on which it was found during this survey. It has also been found associated with the heteropod mollusc *Pterotrachea hippocampus* and with the tunicates *Pegea socia* and *Salpa cylindrica* (Madin & Harbison, 1977).

General remarks

There is still much to be studied with respect to these biological interactions. Some authors suggest that hyperiids associate with hosts only intermittently for food, transportation, or protection (Vader, 1983; Dittrich, 1992). Others argue that these crustaceans are mostly free-living forms (Evans & Sheader, 1972; Westernhagen, 1976), while a third view is that they are obligate symbionts of their hosts and that they are not able to survive independently (Laval, 1980). The range of behavior within these associations is very wide: some species show a parasitoid behavior, leaving their offspring in the host, while other species have a marked and relatively prolonged parental care. Another interesting issue yet to be studied is whether males are more independent from hosts than females. Males are not usually found on the hosts and some hyperiid males even remain unknown (Laval, 1980). We found only one juvenile male (*B. crusculum*) associated with the gelatinous zooplankton examined herein. New investigative efforts could be directed to find out if these symbiotic relations are species-specific or are established between well-defined taxa. This could lead to an improved understanding of the degree of coevolution of hyperiids with their hosts (see Dittrich, 1992). Many unanswered questions remain concerning reproduction and development, fertility, and the impact of parental care on the species success-survival. Further *in situ* observations and direct collections will improve our understanding of these complex and fascinating interactions between phyla.

Acknowledgments

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The scyphomedusan fauna of the Japan Trench: preliminary results from a remotely-operated vehicle

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Key words: ROV, scyphozoa, distribution, physico-chemical parameters

Abstract

A series of dives was conducted during April/May 2002 in a variety of water masses over the Japan Trench using the remotely-operated vehicle ROV *HyperDolphin*. The scyphomedusan fauna was catalogued, resulting in the first records of *Atolla vanhoeffeni* and *Atolla russelli* from Japanese waters. A new genus and species referable to the family Paraphyllinidae was also recognized. The ulmarid scyphomedusa *Poralia rufescens* is by far the most common scyphomedusa in these waters. Its distribution was determined by water mass structure and it was not present in subducted Oyashio Current-derived waters occurring within its usual depth range. Temperature and salinity affected its distribution more than dissolved oxygen concentrations. Distributional information, data on the physico-chemical parameters of the water column, and notes on biological associations with scyphomedusae are introduced.

Introduction

The eastern seaboard of Japan is a highly productive area, due to the presence of upwelling and the formation of frontal zones between various water masses. In this area, warm waters of Kuroshio Current origin meet and mix with the colder nutrient-laden waters of the Oyashio Current before turning eastward and flowing into the open ocean. The species composition and distribution patterns of robust organisms such as krill, shrimps, fishes, and copepods, which can be sampled with conventional plankton nets and midwater trawls, have been studied in this area to a certain extent (e.g., Nishikawa et al., 1995). Much attention has focused in recent years on the physical and biological properties of these water masses, with a special edition of the Journal of Oceanography (Vol. 54, No. 5, 1998) devoted to the topic. Very little information, however, is presently available on the gelatinous macroplanktonic community. Two dives with a crewed submersible off the Boso Peninsula (Pérès, 1959), and a series of dives with the *Mir* submersibles (Vinogradov & Shushkina, 2002 and references therein), are the only exceptions. A cruise (YK02-06) on the R/V *Kaiyo* was designed to characterize the upper water column fauna and identify the physical parameters affecting vertical distributions of the macroplanktonic gelatinous organisms. Particular attention was paid to scyphozoan medusae and their associated fauna.

Materials and methods

A transect that included Oyashio-derived cold waters, Kuroshio-derived warm waters, and their

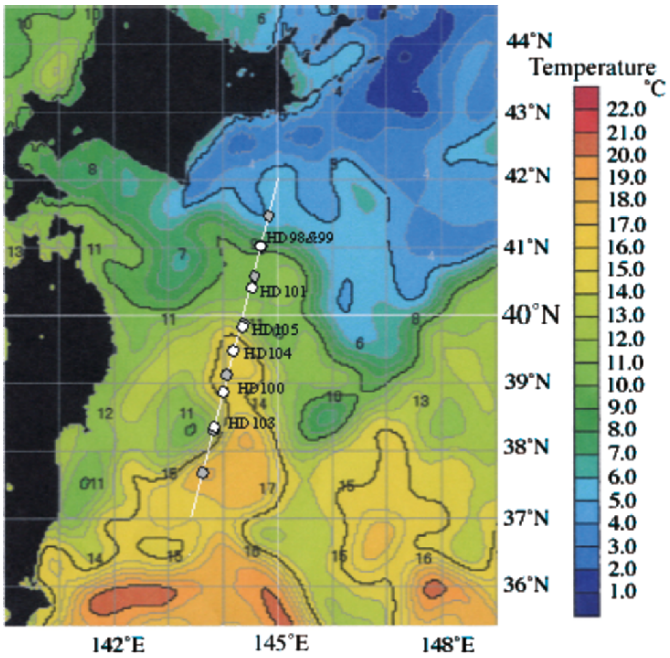

Figure 1. Satellite map of surface water temperatures over the Japan Trench on 29 April 2002 with XCTD (grey circles) and ROV *HyperDolphin* (HD) Dive sites (white circles) superimposed.

respective mixing and frontal zones was run along the eastern seaboard of Japan using the R/V *Kaiyo*. Water masses were identified using satellite imagery and XCTD (eXpendable Conductivity, Temperature and Depth profiling system) probes, and dive surveys using the ROV *HyperDolphin* were carried out to determine the vertical distributions of the macroplanktonic gelatinous organisms (Fig. 1). Dives analyzed during this study were conducted from 22 April to 1 May 2002 in the locations and to the maximum depths outlined below: Dive 98, 41° 00′ N, 144° 41′ E, 1034 m; Dive 100, 38° 56′ N, 143° 06′ E, 1000 m; Dive 101, 40° 26′ N, 144° 32′ E, 2000 m; Dive 103, 38° 20′ N, 143° 55′ E, 1002 m; Dive 104, 39° 30′ N, 144° 15′ E, 1212 m; Dive 105, 39° 52′ N, 144° 22′ E, 1000 m. All dives were conducted during daylight hours with the exception of the latter halves of dives 98 and 103, where the ROV surfaced at 20:31 and 20:35, respectively. Sunset on these two days was at 18:09 and 18:13, respectively.

The ROV *HyperDolphin* was equipped with a high-definition camera integrating an ultra sensitive super HARP (High gain Avalanche Rushing Photo-conductor) tube. Camera sensitivity was F1.8 at 2 lux, gain was variable at 4 – 200 times, the signal to noise ratio was 43 dB, and resolution was 800 TV lines. The zoom lens had a minimum focal length of 5.5 mm and a 5× zooming ratio. There were five 400-W SeaArc HMI/MSR metal halide lamps. Two were situated on the port swinging boom arm, and one on the starboard swinging boom arm. These arms were usually opened such that the lights optimized the field of view of the high-definition camera, but were sometimes moved to optimize lighting when making observations of individual organisms *in situ*. The remaining two lights were forward-pointing and fixed to the frame of the vehicle. Video footage was recorded continuously and simultaneously with depth/time overlay on Sony BCT-D124L Digital BetaCam tapes via an analogue composite signal, and without overlay on BCT-124HDL HDCAM tapes via a native digital signal at 1080i and 30 frames.

Physico-chemical data were collected using a SeaBird SBE19 CTD (Conductivity, Temperature and Depth profiling system) and an SBE13 oxygen sensor attached to the vehicle on all dives. CTD and dissolved oxygen were correlated to the presence of a given animal by matching the time-code on the CTD series to the timecode on video.

Water mass profiles were plotted using linear interpolation, adding CTD data from Dive 99 (41° 00′ N, 144° 42′ E, 1206 m). Specimens were collected for positive identification using a suction sampler. A motor-driven fan slurps the specimen and the water surrounding it into either a 90 mm diameter intake hose and thence through an 80 mm diameter intake hole into a single clear acrylic canister of 300 mm diameter and 285 mm height, or into one of three gate valve samplers. The gate valve sampler, developed at the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) according to a concept outlined by Dr William Hamner, allows collection of larger and more fragile organisms. This sampler uses gate valves, which are sold commercially and designed to control irrigation for agriculture. The gate valve sampler consists of an elongated box-shaped Plexiglas body (35 mmL×18 mmB×18 mmH) which is sealed by a metal plate that blocks the box transversely. This plate slides open and closed and acts as a gate to control the water flow. The plate slips between two rubber rings that act to seal the 103 mm diameter hole. It can be used directly as an aquarium, thereby reducing stress to the captured animal. The frontal wake created by the pressure of forward movement of the sampler is minimized by applying gentle suction with the suction sampler to draw a specimen into the gate sampler before the gates are closed. This system works efficiently and effectively, and we have developed an array of three gate valve samplers with box-shaped bodies for the ROV *HyperDolphin* system. Sampling of voucher specimens allowed recognition of species in the HDTV video record based on their external macromorphology and behaviour. Animals collected in the single cannister of the suction sampler were transferred to shipboard aquaria, phototanks, or planktonkreisels (Hamner, 1990) for behavioural observation and positive identification while those sampled with the gate samplers were observed within the original collection device. Still digital photographs of specimens were taken with a Nikon $D1^H$ digital camera with a macro lens (AF Micro Nikkor 105 mm 1:2.8 D) and recorded in TIFF-RGB format at an image size of 2000 × 1312 pixels. Illumination was provided by National Ref Lamp colour-balanced flood bulbs (PRF-500WB) which lit the specimen from the side before a black felt backdrop. Observations of the live animals were also made in the laboratory under a Nikon SMZ-U dissecting microscope and recorded on DV tape. Sketches were made in cases where the still image and video equipment were unable to resolve or record internal or fine structures to our satisfaction.

Results

Order Coronatae Vanhöffen, 1892
Family Periphyllidae Haeckel, 1880
Genus *Periphylla* Haeckel, 1880
Periphylla periphylla (Péron & Lesueur, 1810)

Description: Coronate medusae with four interradial marginal sense organs, and 12 tentacles arranged in groups of three between adjacent rhopalia; gonads eight, U-shaped; bell steeply pointed with height roughly equivalent to the combined length of the pedalia and marginal lappets; tentacles thick, more or less straight.

Distribution: Observed depth of occurrence ranged from 322 to 750 m. Physico-chemical parameters of the water column habitat were as follows: temperature 2.6–3.8 °C, salinity 33.68–34.20, dissolved oxygen 0.7–5.1 ml/l, sigma-t 26.87–27.21 kg/m^3. The observed distribution of *Periphylla periphylla* is outlined in Figure 2. All records of *Periphylla* occurred during daylight hours, except for a single record at 350 m depth at 19:51 on Dive 103.

Comments: None of the individuals were observed in association with other organisms.

Family Paraphyllinidae Maas, 1903

A new member of this family recognized in Japanese waters awaits a full description and taxonomic treatment (see below). We have yet to identify characters that would allow easy *in situ* identification at the family level. Waving the manipulator arms of the vehicle to create gentle currents sometimes changed the attitude of this medusa enough to peek under the exumbrella and ascertain whether the four rhopalia were situated perradially or not – the rhopalia count and perradial condition being definitive for the family. The sole genus currently recognized in this family is *Paraphyllina* Maas, 1903, containing two species – both with 12 tentacles arranged in groups of three (Maas, 1903; Russell, 1956).

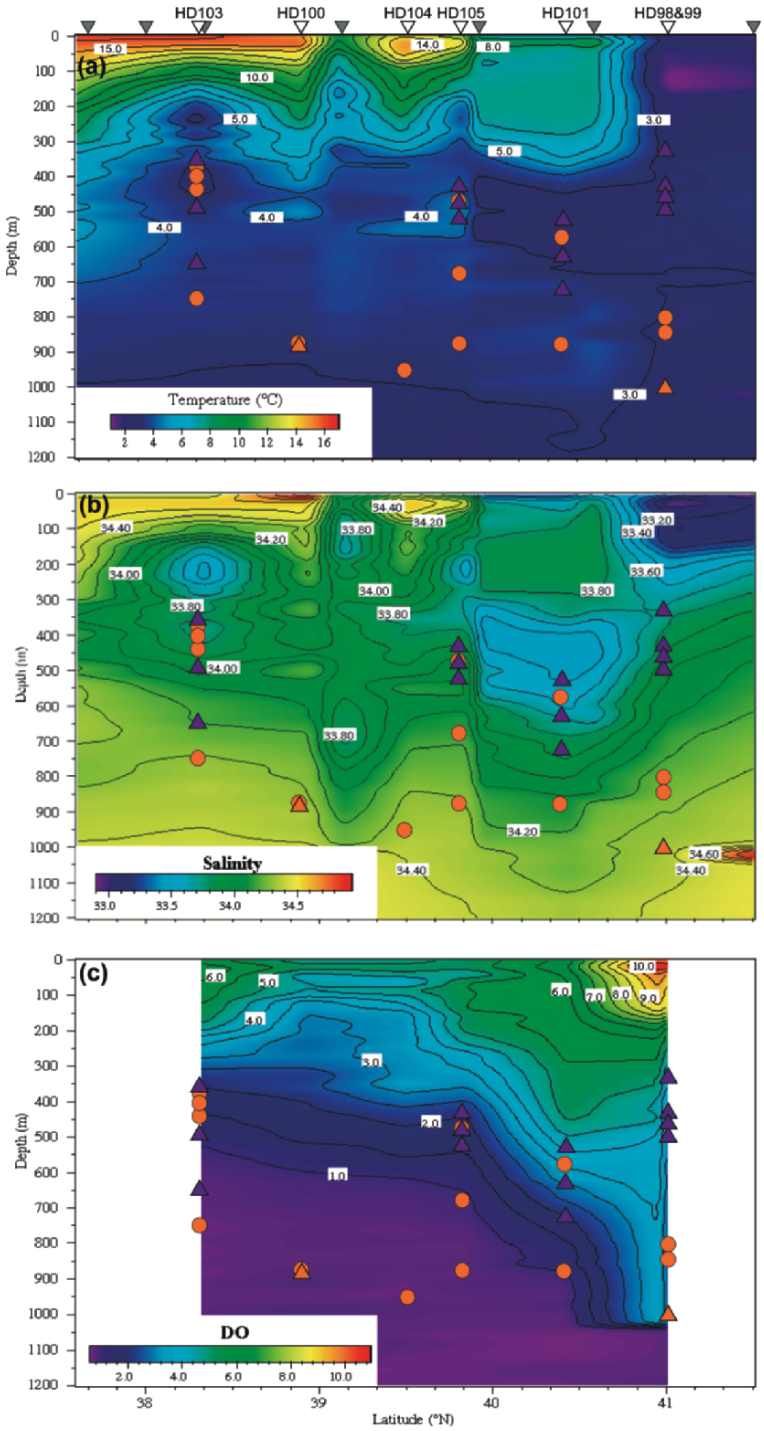

Figure 2. Horizontal and vertical distributions of the coronate scyphomedusae *Periphylla periphylla* (purple triangles), *Atolla* spp. (orange circle), and the undescribed paraphyllinid species (orange triangles) over the Japan Trench during April/May 2002, and correlations with (a) water temperature, (b) salinity, (c) and dissolved oxygen (DO) concentrations. Upturned grey triangles signify latitudes at which XCTD probes were deployed and upturned white triangles where ROV *HyperDolphin* (HD) dives were conducted.

Genus and species undefined
Common Japanese name: *benimanjukurage* (red manju medusa)

Description: Paraphyllinidae with 20 tentacles arranged in groups of five, and with four perradial rhopalia; rhopalia with no ocellus or lens present, and no opaque pigmented bulbous collar. Recognizable *in situ* by reddish brown pigmentation over the whole body such that none of the internal structure is visible, an extremely pronounced coronal groove, four bright white statocysts with five tentacles between each, a shallow conical bell of height equivalent to the combined length of the pedalia and marginal lappets when the medusa is floating in a stationary position, and tentacles folded towards the centre of the subumbrella or extruded only from the opening formed by the extremely long lappets when they are folded in as the medusa is stationary. Macromorphology is shown in Figure 3.

Distribution: Observed depth of occurrence was at 880 and 996 m. Physico-chemical parameters of their water column habitat were as follows: temperature 2.7–3.2°C, salinity 34.31–34.33, dissolved oxygen 0.7–0.8 ml/l, sigma-t 27.32–27.38 kg/m^3.

Comments: No organisms have yet been observed in association with this medusa.

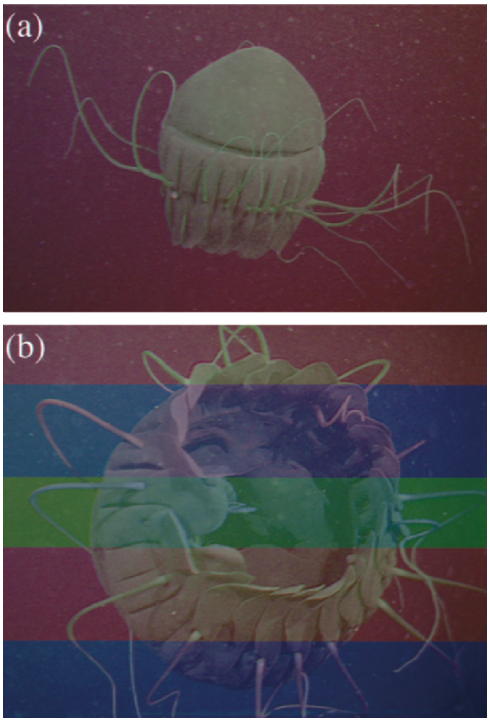

Figure 3. (a) Side view and (b) oral view of the paraphyllinid coronate scyphomedusae referred to as the red manju jelly (*benimanjukurage*).

Family Atollidae Bigelow, 1913
Genus *Atolla* Haeckel, 1880

Recognizable *in situ* by dark red, reddish brown to black pigmentation on manubrium, a flattened and often heavily pigmented bell, and by one very long hypertrophied tentacle dragged behind the umbrella as they swim. Several individuals had a single small crustacean associated with the centre of their exumbrella. The observed distribution of the genus *Atolla* is outlined in Figure 2.

Atolla wyvillei Haeckel, 1880
Common Japanese name: *murasakikamurikurage (purple coronate medusa)*

Description: Adults can be recognized by the primrose-shaped stomach base, lack of pigment spots between each gonad and the stomach base, large size, deeply furrowed bell, lack of papillae on lappets, and presence of auriform gonads.

Distribution: Below 500 m.

Atolla vanhoeffeni Russell, 1957
Common Japanese name: *batsukamurikurage (cross coronate medusa)*

Description: Recognizable *in situ* by its clear bell, 20 tentacles, cross-shaped stomach base, and presence of eight pigment spots situated between each gonad and the stomach base.

Distribution: Positive identification at 484 m depth (Dive 103, 38° 20′ N, 143° 55′ E, temperature 3.78 °C, salinity 34.06, dissolved oxygen 1.23 ml/l, sigma-t 27.06 kg/m^3).

Comments: A specimen captured in Sagami Bay (*Shinkai 2000* Dive 1139, 35° 01′ N, 139° 22′ E, depth 711 m, temperature 4.45 °C, salinity 34.02, dissolved oxygen 1.50 ml/l, sigma-t 26.96 kg/m^3) was used to verify our identification based on the video record and, as it represents an extension of the known geographical range, has been placed in the National Science Museum, Tokyo, under

registration number NSMT-Co 1401. Another individual, also collected from Sagami Bay (*Shinkai 2000* Dive 1055, 35° 01′ N, 139° 22′ E, depth 557 m, temperature 5.35 °C, salinity 34.56, dissolved oxygen 1.87 ml/l, sigma-t 27.28 kg/m^3), has been stored at JAMSTEC under registration number MP1-4 024273.

Atolla russelli Repelin, 1962
Common Japanese name: *girishajuujikurage (Greek cross medusa)*

Description: Recognizable *in situ* by deep red bell and skirt pigmentation (largely obscuring gonads), bell vaulted with deep radial grooves, 16–20 tentacles, and squarish Greek cross-shaped stomach. An individual with the characteristics outlined below was sampled. Mature female; bell diameter 19 mm; 20 tentacles; 20 deep radial grooves on bell; eight gonads hanging freely in the subumbrellar cavity surrounding the stomach but hidden behind the deeply red-pigmented dermis.

Distribution: Sampled at 900 m depth on *HyperDolphin* dive 103 (38° 20′ N, 143° 55′ E, temperature 2.95 °C, salinity 34.37, dissolved oxygen 0.68 ml/l, sigma-t 27.39 kg/m^3, 20 tentacles). Other positive records where the quality of the HDTV imagery left no doubt as to species identification occurred at 1146 m (Dive 104, 39° 30′ N, 144° 15′ E, temperature 2.69 °C, salinity 34.44, dissolved oxygen 0.64 ml/l, sigma-t 27.47 kg/m^3, 20 tentacles).

Comments: As this record represents an extension of the known geographic range, the specimen has been placed in the National Science Museum, Tokyo, under registration number NSMT-Co 1402.

Family Nausithoidae Bigelow, 1913
Genus *Atorella* Vanhöffen, 1902
The genus is recognizable *in situ* by the presence of six tentacles, six rhopalia, and a coronal groove.

Atorella sp.
Common Japanese name: *mutsuashikamurikurage (six legged coronate medusa)*

Description: Recognizable *in situ* by a combination of characters including six tentacles, six rhopalia, eight cream-tan gonads, and a heavily red–brown pigmented stomach wall.

Distribution: This species was found in Suruga Bay at a depth of 1038 m with physico-chemical parameters as follows: temperature 3.22 °C, salinity 34.45, dissolved oxygen 1.42 ml/l, sigma-t 27.43 kg/m^3.

Comments: This medusa is provisionally listed here as a possible inhabitant of waters off Sanriku, as water masses with these physico-chemical properties occur in the Kuroshio Current extension area.

Order Semaeostomeae Agassiz, 1862
Family Ulmaridae Haeckel, 1879
Subfamily Poraliinae Larson, 1986
Genus *Poralia* Vanhöffen, 1902
Poralia rufescens Vanhöffen, 1902
Common Japanese name: *ringokurage* (apple medusa)

Description: Poraliinae with 30 marginal tentacles and 15 rhopalia arranged in the following sequence, where T = tentacle and S = statocyst: STTTSTTTTSTTSTSTSTSTTSTTSTSTTSTTSTTSTTTSTTSTT. All lappets similar in length and rectangular in outline, with deep cleft in rhopalial lappets and shallow cleft in tentacular lappets; radial canals wide, unbranched (29 of 31 [33] total; one canal branched into two distally, and two canals joined into one distally), leading to a wide peripheral ring canal and usually continuing into each lappet to meet the tentacle bases or rhopalia. Disk diameter 90 mm. Stomach circular, occupying just under half disk diameter. Juvenile specimen? with no visible gonads. Marginal tentacles orange, tapering to a white tip. Exumbrella covered in nematocyst warts. Epidermis reddish–brown except for whitish oral surface of the six oral arms, which are V-shaped in cross section. Partially digested shrimplike animal of 9 mm carapace length, round eyes of 1.5 mm diameter and with reduced rostrum found in stomach.

Distribution: The observed distribution of *Poralia rufescens* is outlined in Figure 4. Physico-chemical parameters of their water column habitat were as follows: temperature 2.7−3.6 °C, salinity 34.02−34.41, dissolved oxygen 0.6−3.8 ml/l, sigma-t 27.09−27.43 kg/m^3.

Comments: A single species of lysianassid amphipod was commonly associated with *Poralia* medusae over the Japan Trench. They were usually

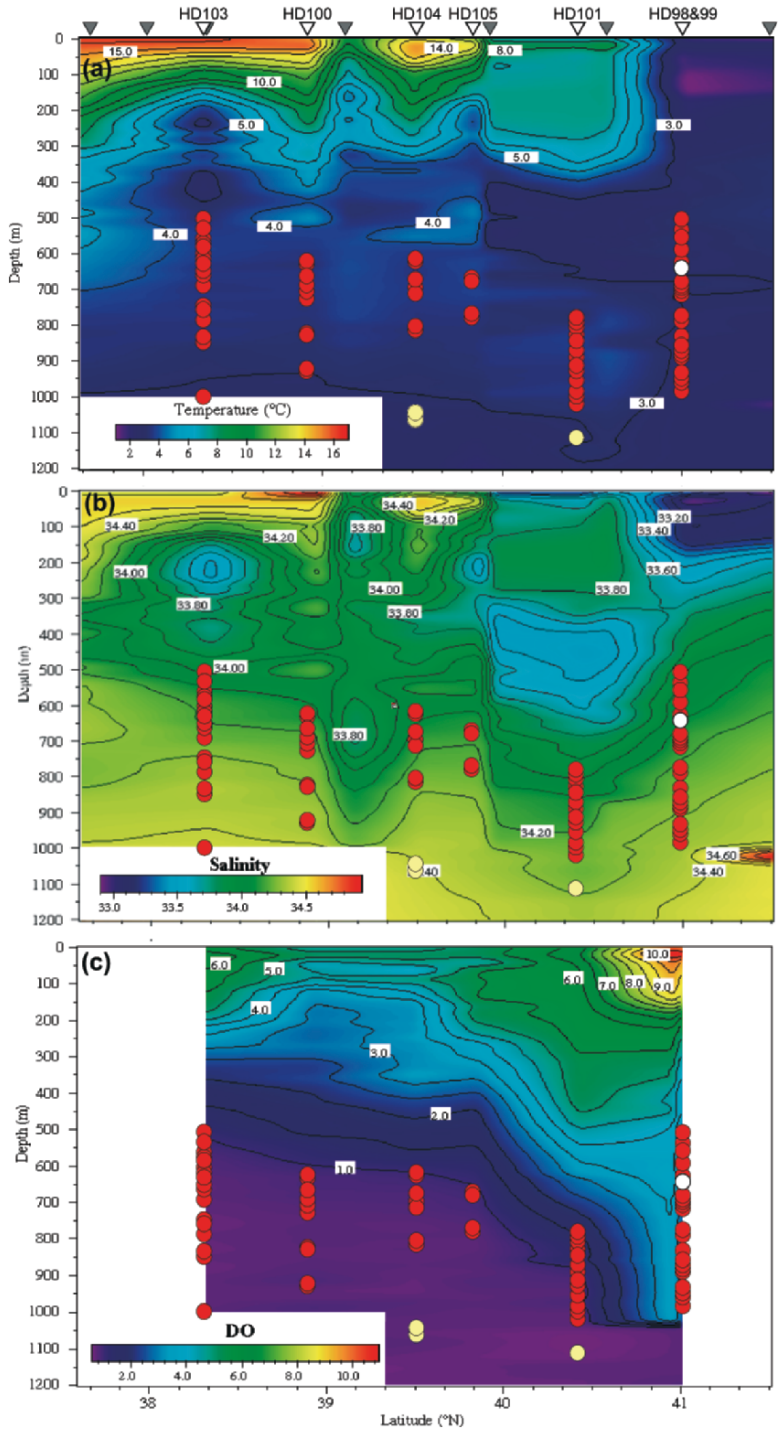

Figure 4. Horizontal and vertical distributions of the ulmarid scyphomedusae *Poralia rufescens* (red circles), *Deepstaria enigmatica* (white circle), and *Tiburonia granrojo* (pink circles) over the Japan Trench during April/May 2002, and correlations with (a) water temperature, (b) salinity, and (c) dissolved oxygen (DO) concentrations. Upturned grey triangles signify latitudes at which XCTD probes were deployed and upturned white triangles where ROV *HyperDolphin* (HD) dives were conducted.

attached at the bases of the oral arms but sometimes elsewhere on the subumbrella and rarely on the exumbrella. Between one and six amphipods were associated with each medusa. The above description of the Japanese morphotype is based on a specimen collected in Suruga Bay on *Shinkai 2000* Dive 1335 at a depth of 867 m. It was in much better condition than the four specimens taken over the Japan Trench. Although *Poralia* sp. has been reported previously from Japanese waters (Hunt & Lindsay, 1999), this is the first identification to species level. The specimen has therefore been placed in the National Science Museum, Tokyo, under registration number NSMT-Co 1403.

Subfamily Deepstariinae Larson, 1986
Genus *Deepstaria* Russell, 1967
Deepstaria enigmatica Russell, 1967
Common Japanese name: diipusutariakurage (deepstar medusa)

Description: Recognizable *in situ* by the lack of marginal tentacles, the presence of five slender oral arms that do not extend beyond the bell margin, the bell being transparent white throughout except for some brown pigmentation around bell margin, and the thin anastomosing gastrovascular canals.

Distribution: Sampled at 669 m depth on *HyperDolphin* dive 98 (41° 00′ N, 144° 41′ E, temperature 2.93 °C, salinity 34.13, dissolved oxygen 3.54 ml/l, sigma-t 27.20 kg/m^3).

Comments: two anuropid isopods inhabited the inner surface of the bell close to the oral arm bases, as previously reported for other Japanese specimens (Lindsay et al., 2000). Although *Deepstaria enigmatica* has been reported previously from Japanese waters (Lindsay et al., 2000), this is the first specimen collected and it has therefore been placed in the National Science Museum, Tokyo, under registration number NSMT-Co 1404.

Subfamily Tiburoniinae Matsumoto, Raskoff & Lindsay, 2003
Genus *Tiburonia* Matsumoto, Raskoff & Lindsay, 2003
Tiburonia granrojo Matsumoto, Raskoff & Lindsay, 2003
Common Japanese name: *yubiashikurage* (finger-foot medusa)

Description: Recognizable *in situ* by the lack of marginal tentacles, the presence of four to seven thick, blunt oral arms that extend beyond the bell margin to a length of no more than the height of the bell, pigmentation red–brown throughout, nematocyst warts covering all surfaces, and thin, anastomosing gastrovascular canals.

Distribution: Sampled at 1019 m depth on *HyperDolphin* dive 99 (41° 00′ N, 144° 42′ E, temperature 2.66 °C, salinity 34.36, dissolved oxygen 0.65 ml/l, sigma-t 27.40 kg/m^3). The observed distribution of *T. granrojo* is outlined in Figure 4.

Comments: No organisms were observed in association with these medusae. Although *Tiburonia granrojo* has been reported previously from Japanese waters (Matsumoto et al., 2003), this is the first specimen collected and it has therefore been placed in the National Science Museum, Tokyo, under registration number NSMT-Co 1405.

Discussion

Order Coronatae Vanhöffen, 1892

The genera *Periphylla* and *Atolla* are arguably the best recognized of the midwater scyphomedusae. Only one species in the genus *Periphylla* is currently considered valid but, based on the morphotypes we have observed and/or sampled from submersibles and the current trend towards resurrection of previously described scyphozoan species based on molecular data (Schroth et al., 2002), we believe that there may be at least one cryptic species within this nominal species. *Periphylla* has been reported previously from the 250–1000 m depth layer in the Kurile–Kamtchatka region (Vinogradov & Shushkina, 2002), corresponding most closely with *HyperDolphin* Station 98 in our survey, and from 504 m in Sagami Bay (Hunt and Lindsay, 1999). No evidence of vertical migration was found for any of the scyphomedusan species observed in this study. However, this may be an artifact of the early retrieval time of the ROV (i.e. only 2 h 20 min after sunset) during the two dives that contained a nighttime component.

Atolla wyvillei Haeckel, 1880, is the only currently recognized species of *Atolla* reported previ-

ously from Japanese waters (Kramp, 1961). *In situ* identification to species level of members of the genus *Atolla* is often difficult, especially in smaller specimens, without sampling or extensive high definition video footage being taken. Many observational records allowed the elimination of some species from consideration (e.g. not *vanhoeffeni* or *russelli*, with 30 tentacles, no papillae, probably *wyvillei*; transparent, tentacle number between 20 and 24, either *vanhoeffeni* or a young *wyvillei* but viewing angle did not allow observation of the eight pigment spots) but did not allow for positive identification of the specimen. The shape and length of the radial septae are usually not discernable in situ, therefore confounding identification based on presently available taxonomic keys.

Atolla vanhoeffeni Russell, 1957, and *A. russelli* Repelin, 1962, are both recorded for the first time from Japanese waters, although *A. russelli* has been observed in north-east Pacific waters (M. Arai, personal communication) The present specimen of *A. russelli* differs in several ways from the original description by Repelin (1962), and correlates more closely with the description of *A. russelli* by Pages et al. (1992) of a single specimen from the Benguela Current. The radial septae are straighter than those shown in the original figure of *A. russelli* (Repelin, 1962), curving in slightly at the ends as described by Kramp (1968) and Pages et al. (1992). Contrary to previous descriptions, the tips of the radial septae in the present specimen extend just past the edge of the coronal muscle. Although the gonads hang freely in the subumbrellar cavity surrounding the stomach, as described by Repelin (1962) and differing from any other currently recognized species of *Atolla*, they are not lobed but rather rectangular in shape. Pages et al. (1992) also reported gonad shape to be rectangular but Kramp's (1968) specimen was too damaged to ascertain their shape or structure. The tentacle number in the present specimen was 20, not 16–18 as reported in previous studies. However, tentacle number has been shown to not always be a reliable character for distinguishing between different species of *Atolla* (Mauchline & Harvey, 1983). We therefore record this species as *A. russelli* pending the sampling of at least one other specimen from Japanese waters and re-examination of Repelin's original material to determine whether the present specimen instead warrants the erection of a new species.

Most of the observations of individuals of *Atolla* with clear exumbrellas (i.e., *Atolla vanhoeffeni*) occurred at depths shallower than 500 m. Juveniles of *A. wyvillei* Haeckel, 1880, were also sampled and they too have clear bells. It is necessary to obtain close-up video footage either of the dorsal or ventral surface to enable distinction from *A. vanhoeffeni* based on the lack of pigment spots and shape of the stomach base. Tentacle count is greater in juvenile *A. wyvillei*, gonad size is smaller relative to bell diameter, and there is little dark pigmentation around the rim of the bell, in contrast with *A. vanhoeffeni* (personal observation). *Atolla parva* also has a clear non-pigmented bell but should be distinguishable based on tentacle count, lappet shape and gonad size/morphology. *Atolla parva* has yet to be recorded from Japanese waters. Species of *Atolla* have been reported previously in Sagami Bay, with nine individuals at depths between 555 and 775 m, and a single individual at 1167 m depth (Hunt & Lindsay, 1999). Records of *Atolla* also occur in Pérès (1959) from 38° 07′ N, 142° 16′ E, at depths of 580–890 m and temperatures of 3–5 °C, but no photographic plates were published so the identifications cannot be checked.

The genus *Atorella* contains only one species with eight gonads, *Atorella octogonos* (Mills et al., 1987). The *Atorella* individual observed in Suruga Bay had eight gonads but also had a heavily pigmented stomach wall, unlike the transparent stomach of *Atorella octogonos*. The only other record of *Atorella* from Japanese waters is for the polyp of *Atorella japonica* at depths of 40–60 m off Okinoshima Island, Japan Sea (Kawaguti & Matsuno, 1981). The morphology of the adult medusa of *Atorella japonica* is unknown.

Order Semaeostomeae Agassiz, 1862

A single morphotype of *Poralia* has been recognized in Japanese waters. Only one species is currently recognized in this genus, *Poralia rufescens* Vanhöffen, 1902, but evidence suggests that multiple species are contained under this name (Larson, 1986). At least two undescribed species have been distinguished (Larson et al., 1991; Larson et al., 1992; Wrobel & Mills, 1998) but descriptions

are as yet unpublished. The Japanese species exhibits the characters of *P. rufescens* Vanhöffen, 1902, as currently described (Russell, 1962; Mianzan & Cornelius, 1999). Minimum bottom depth along the survey line was always deeper than 4000 m, proving a midwater, rather than a benthopelagic, habitat for this medusa in contrast with findings by Smith (1982) but agreeing with Larson (1986) and Larson et al. (1992). Benthopelagic occurrences of *Poralia* may well be artifacts of the sea floor occurring within the regular vertical distribution range of this medusa. Distributional records suggest that *Poralia* inhabits low temperature waters (Figure 4a) of intermediate salinity (Figure 4b) and is not affected by dissolved oxygen concentrations (Figure 4c). Depression of distributional depth seemed to occur as a result of the subduction of low-salinity waters derived from the Oyashio Current system (Figure 4b). The current *Poralia* morphotype has been reported previously from Sagami Bay at depths of 614, 750, 835, 857 and 1369 m (Hunt & Lindsay, 1999). Accompanying physico-chemical parameters at those depths in Sagami Bay were as follows: temperature 2.5–4.9 °C, salinity 34.24–34.48 and dissolved oxygen 1.1–1.8 ml/l. The same *Poralia* morphotype also occurs in plate 4 of Pérès (1959) under the misnomer *Aequorea* where it is reported from 38° 07′ N, 142° 16′ E, at a depth of 700–750 m and temperature of 3–4 °C. The lysianassid amphipod may be a good *de facto* indicator of *Poralia* occurrence as no free swimming individuals were observed. Records exist for ?*Poralia* at depths of 900–3000 m and for ?*Stygiomedusa* from 750 to 1750 m depth in the Kurile–Kamchatka region (Vinogradov & Shushkina, 2002). However, no reference was made to either a *Tiburonia* or a paraphyllinid form and the authors themselves appended a question mark to their identifications. Accordingly, we are somewhat circumspect as to the true identities of these large reddish–brown medusae.

Several observations of associations between anuropid isopods and *Deepstaria enigmatica* have been reported (Barham & Pickwell, 1969; Lindsay et al., 2000; S.H.D. Haddock, personal communication). Three specimens of *Anuropus bathypelagicus* Menzies & Dow, 1958, the species reported to occur in association with *D. enigmatica* (Barham & Pickwell, 1969), have been caught in otter trawls (mesh aperture 18–30 mm) from depths of 600–700 m over the Japan Trench (Saito et al., 2002). Although all three specimens of *Deepstaria enigmatica* that we have observed to date have contained a pair of anuropid isopods within their bells, we have yet to observe free-living anuropids during over 300 h of submersible surveys in Japanese waters. It is quite possible that the distributions of anuropid isopods could be used to infer distributional patterns of *Deepstaria enigmatica*, because the medusa we sampled was observed at 669 m depth, agreeing well with the distribution of 600–700 m depth for *Anuropus bathypelagicus* reported by Saito et al. (2002). No organisms have so far been observed to occur in association with the medusa *Tiburonia granrojo* – a medusa recently described as the first member of its subfamily based partly upon samples and images collected during the present research survey (Matsumoto et al., 2003).

Conclusions

The ulmarid scyphomedusa *Poralia rufescens* is by far the most common scyphomedusa in waters over the Japan Trench. Its distribution was determined by water mass structure and it was not present in subducted Oyashio Current-derived waters occurring within its usual depth distribution range. Temperature and salinity affected its distribution more than dissolved oxygen concentrations. More data are necessary to characterize the physico-chemical parameters determining the distributions of other members of the scyphomedusan fauna in the study area. The discovery of a putatively new genus of coronate scyphomedusa and a new subfamily of ulmarid scyphomedusa points to our general lack of knowledge about gelatinous deep sea organisms.

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A golden age of gelata: past and future research on planktonic ctenophores and cnidarians[☆]

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Key words: siphonophore, jellyfish, bioluminescence, biodiversity, blue-water diving, submersible

Abstract

The study of the natural history of gelatinous zooplankton ('gelata') reached a high point at the end of the 19th century, when scientists first began to understand the phylogenetic and ecological links between cnidarians and ctenophores. Siphonophores, carefully figured in their entirety, and gauze-like lobate ctenophores too fragile to touch, were described by the dozens. In the ensuing years, focus on zooplankton shifted toward more 'industrial' goals such as quantitative sampling using plankton nets. While plankton scientists were busy summing tattered parts, they lost sight of the whole jellies themselves, and a crustaceocentric view of the ocean came to dominate. During this period, the most dramatic breakthroughs in cnidarian research came from laboratory studies of neurobiology, physiology, and development, particularly of certain model organisms. Now, at the turn of this century, we have the opportunity to bring gelata back into primacy. Submersibles and remotely operated vehicles allow us to study entire life histories of organisms that we did not even know existed. The tools of molecular biology allow us to answer questions about development, evolution, and phylogeny that had reached a stalemate. Even in the surface waters, where it might be thought that there is little left to learn, *in situ* observations have revealed unexpected interactions and hidden diversity. The critical roles that these organisms play in the health of the oceans, their position at the crux of many evolutionary debates, and the tools for biotechnology that they provide, have led to resurgent public appreciation and awareness. Although advanced tools do not necessitate good science, we have few excuses for failing to bring about another golden age of gelata.

Introduction

The name 'coelenterata' has long been employed as a convenient way to describe organisms from the two phyla Ctenophora and Cnidaria. Because it implies a degree of relatedness which is now considered inaccurate, this word is typically avoided in a rigorous taxonomic or phylogenetic context. An alternative term – more encompassing but with less implication of shared common ancestry – is the phrase 'gelatinous zooplankton'. This term has the advantage of emphasizing the convergent features of transparency, fragility, and planktonic existence that unite these disparate creatures, without the taxonomic baggage associated with other terms. On the other hand, it is a polysyllabic mouthful. Here the term *gelata* is offered as a collective noun to describe the polyphyletic assemblage of medusae, siphonophores, ctenophores, radiolarians, urochordates, molluscs, and worms that eke out a planktonic existence (Fig. 1). It is meant to have no taxonomic implication, but rather to evoke these diverse groups of non-crustacean macroplankton that are too fragile to sample with conventional oceanographic methods.

[☆]A plenary address of the 7th International Conference on Coelenterate Biology.

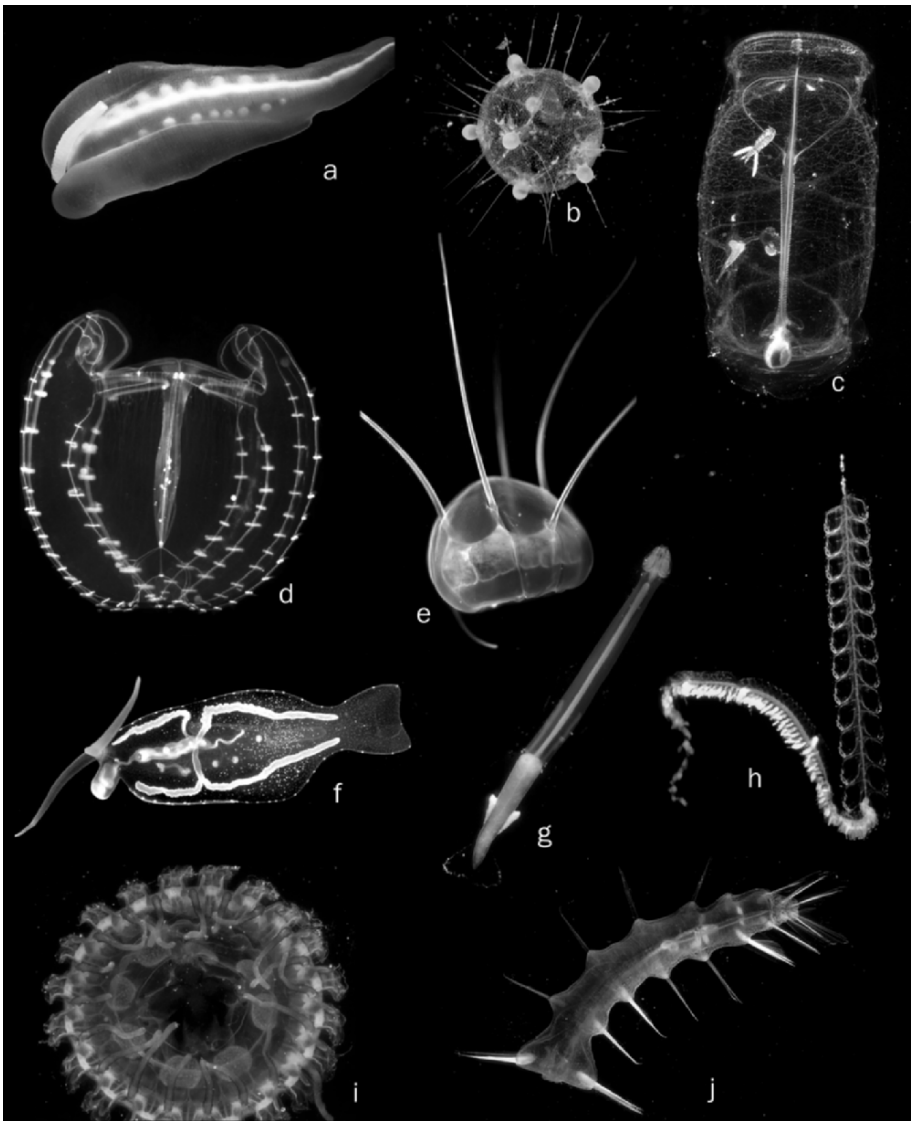

Figure 1. Representative members of gelatinous zooplankton. Organisms from at least eight phyla are included among the gelata. (a) Nemertean. (b) Phaeodarian radiolarian. (c) Salp with parasitic copepod. (d) Lobate ctenophore. (e). Narcomedusan hydrozoan. (f) Nudibranch mollusc. (g). Chaetognath. (h) Physonect siphonophore. (i) Coronate scyphozoan. (j) Polychaete.

The first golden age

Two of the most prominent yet under-appreciated members of the gelata are siphonophores (Cnidaria, Hydrozoa) and comb jellies (Ctenophora). At the end of the 19th century, these taxa occupied more conspicuous positions in both public and scientific consciousness (Winsor, 1972). Comb jellies had several champions in the early 1900s; between 1900 and 1909, twice as many ctenophore species were described than in any other ten-year period before or since (Fig. 2). The observations conducted by Chun (1880) are so unique that only in the last 20 years have some of his species been 're-discovered.'

Because of their apparently intermediate position between benthic hydroids and planktonic medusae, siphonophores elicited a special fascination from those grappling with the implications and mechanisms of Darwinian evolution. The

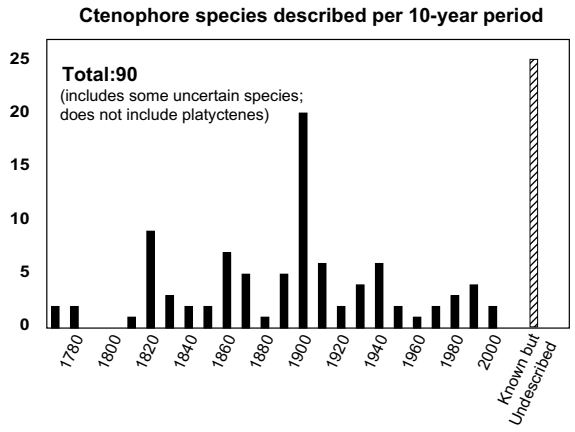

Figure 2. Ctenophores described by decade. The majority of known ctenophore species were described around 1900, with the maximum occurring from 1900 to 1909. Presently there many known deep-sea species, 70% of which are cydippids, which await description. Numbers adapted from Mills (1998), with unpublished data from Harbison, Mills, Haddock, Matsumoto, Lindsay.

great biologists of the time, including Huxley, Haeckel, Vogt, Leuckart, Agassiz, and Darwin himself, enthusiastically enlisted siphonophores in their debates. Deservedly, all these pioneers have had multiple species of gelata named after them.

Illustrations from this time were inspired as much by the beauty of the organisms as by functional scientific interpretation. Haeckel, fresh from his monumental and still unsurpassed treatise on Radiolaria (1887), created detailed illustrations showing entire siphonophore colonies (1888, 1904) with the apparent intent of demonstrating how such diverse specialized parts could serve a united function (Fig. 3b). His representations from this era compare favorably with living specimens captured by a submersible (Fig. 3a). Another champion of siphonophores, Huxley (1856), asserted that 'living nature is not a mechanism but a poem.' Although some artwork of this time has been said to be distorted by ideological predispositions (e.g. Mechnikov, 1874, as cited in Winsor, 1972), these criticisms cannot detract from their many important positive effects: The predominant attitude was a concern with the whole animal, not merely its parts. This is linked to the desire to understand the origins of life, and the perceptions that cnidarians and ctenophores played key roles in answering basic questions of evolution.

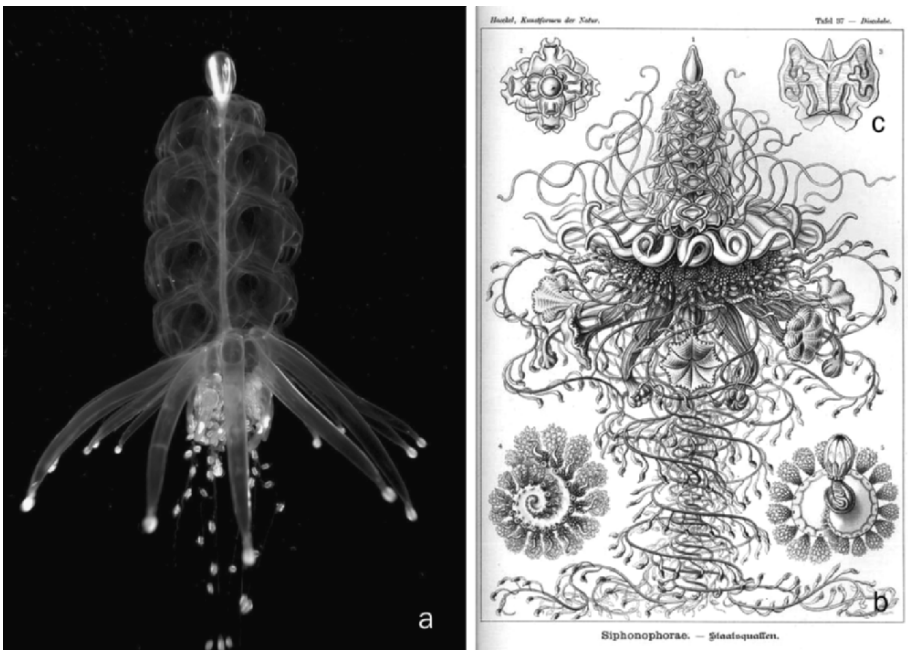

Figure 3. Two views of the siphonophore *Physophora*. (a) Photo of a live specimen collected by submersible. (b) Illustration of whole colony by Haeckel (1904). (c) Detail of isolated nectophore.

Public awareness and fascination with cnidarians followed suit. Haeckel and others presented siphonophores in popular lectures to general audiences. Best-selling books of the time were natural history treatises of Wood (1890), whose encounter with a scyphomedusa inspired a Sherlock Holmes mystery, and Gosse (1853), who described a species of bioluminescent hydromedusa and coined the term 'aquarium' while helping fuel the Victorian aquarium craze with his how-to guides. Siphonophores, medusae, and even radiolarians were depicted in popular glass sculptures by the Blaschkas, who strived for a *tromp l'oeil* degree of realism. Intact living specimens could be obtained because the pace of the sampling, at times using buckets over the side of sailing ships, or dipping from the sea at Mediterranean laboratories, allowed it.

The 'industrial' age

Two shifts occurred in the mid-1900s that contributed to the stagnation of research on gelatinous zooplankton. Ships became larger and faster, and scientists' interest turned to important global questions such as carbon flux. These oceanographic-scale cruises led scientists to ask of the jellies 'how many?' and 'how much?' before having answered 'who?' and 'how?'

In contrast to the drawings of the late 1800s, the depictions of isolated siphonophore bits by mid-20th-century taxonomists show only dissociated parts (Fig. 1c). This was by necessity, as researchers, forced to use nets and other conventional sampling devices, could not obtain intact specimens (cf. Alldredge, 1984; Pugh, 1989; Harbison, 1992; Dennis, 2003). Although this piece-by-piece research is an integral part of our present knowledge (e.g. Totton, 1965), it sheds little light on how the intact organism actually functions.

The situation was worse in lobate ctenophores, where there were not even pieces available for examination. During this period, it seems that if an organism could not be fixed in formalin, it was not studied. Figures in textbooks have been copied generation after generation without reference to living animals, like a visual game of '*Telephone*.' A result of this piecemeal approach to examining gelata is that even invertebrate zoologists had very little understanding of the organisms themselves. For example, the best-known ctenophore genus, *Pleurobrachia,* is depicted upside-down in a majority of scientific and educational illustrations. Although the early taxonomists began this unfortunate convention, there has been little effort to correct it in the intervening 100 years. This seems like a harmless and arbitrary choice, but depicting comb jellies as though they encounter the world with their aboral end perpetuates false affinities with medusae, and impairs our understanding of their distinct non-jellyfish-like modes of feeding (Greve, 1970; Harbison et al., 1978; Moss & Tamm, 1986).

Another result of the difficulty of sampling ctenophores is that their diversity has been underestimated. Three genera (*Pleurobrachia*, *Beroe*, *Mnemiopsis*) have received the vast majority of attention because they are common in shallow coastal waters or because they are robust enough to come up in plankton tows. The lobate genus *Bathocyroe* is extremely abundant throughout the deep ocean – arguably more common than any of the better known genera – yet it was only described 25 years ago (Madin & Harbison, 1978a). The diversity of ctenophores and planktonic cnidarians seems to reside in the higher taxonomic levels. Rather than a few speciose genera, these phyla have many monotypic genera, and families or even orders with only a handful of members. Within the comb jellies, the diversity is demonstrated in the variety of body shapes, sizes, and feeding specializations. Nearly every permutation possible (with or without lobes, auricles, tentacles, tentilla) occurs within the phylum (Fig. 4).

At least in the ctenophores there has been some representation of their many body plans; siphonophores are much more poorly depicted. The aberrant cystonect *Physalia* is generally used to represent the group, although it possesses few traits in common with any other siphonophore genus. (This is similar to the use of *Obelia* as the 'textbook' example of hydromedusan life-cycles, when it is one of the least representative members of the group.) Although it should be rectified, this bias is understandable given that the truly abundant siphonophores are fragile, transparent, and difficult to collect, whereas in certain regions the Portuguese Man o'War, like the ctenophore *Pleurobrachia*, can be washed conspicuously onto beaches.

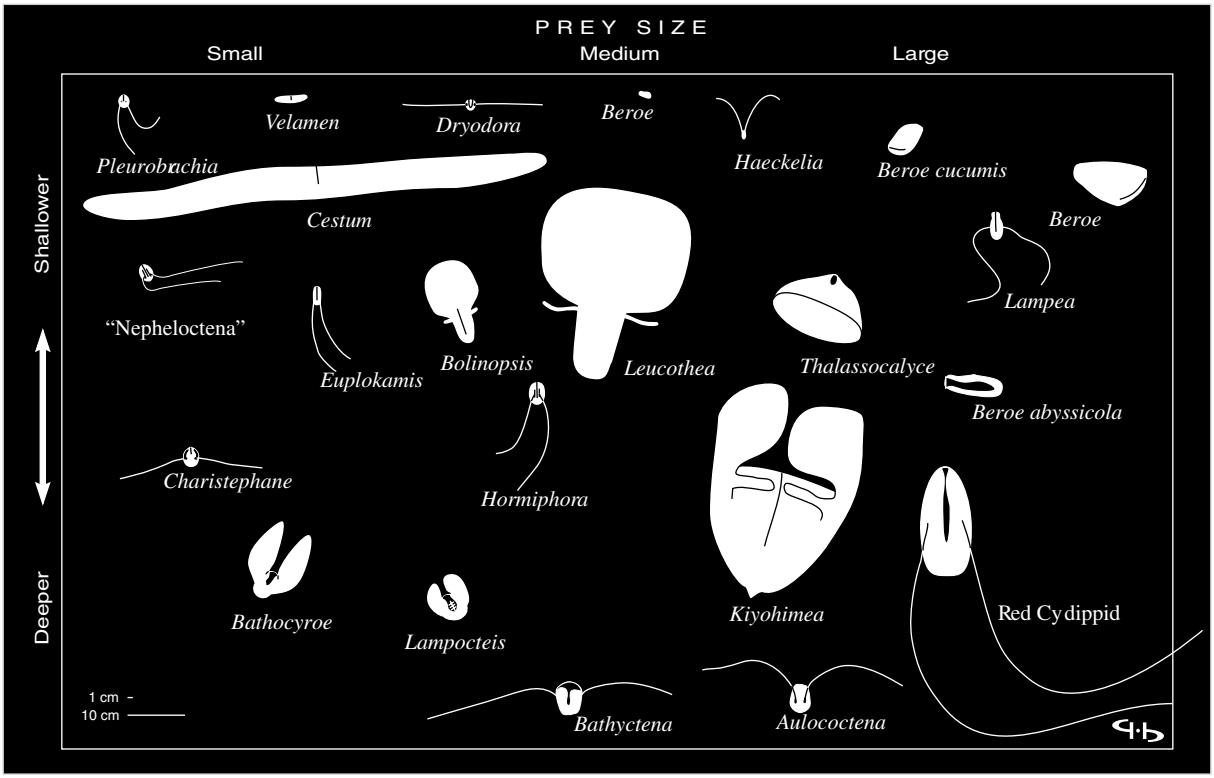

Figure 4. Diversity of ctenophore shapes and sizes. Ctenophores range in size from a few millimeters to nearly a meter. They are found to great ocean depths and eat a range of prey types, from small copepods to shrimp, and even other jellies.

A new golden age

Are we indeed poised for a resurgence in interest and understanding of gelata? There are several reasons why this is an opportune time for significant advances in our understanding of these creatures.

Collection techniques

With the exception of pioneers like Bieri (1966) and Ceccaldi (1972), few people attempted to observe plankton in their natural environment before Hamner and students pioneered the technique of blue-water SCUBA diving (Hamner, 1975; Hamner et al., 1975). This is a system of tethers and procedures for making *in situ* observations on water-column organisms and processes. Although it can not be said to be commonplace, this method has found broad applicability in examinations of marine snow (Silver et al., 1978; Alldredge & Silver, 1988), microbes (Delong et al., 1993), diatoms and cyanobacteria, in addition to gelata (Harbison et al., 1978; Haddock & Case, 1999). This intimate view of the shallow open water has been extended to the deep-sea by the use of submersibles and ROVs (e.g. Pugh & Harbison, 1987; Widder et al., 1989; Hamner & Robison, 1992; Lindsay et al., 2000). In addition to improved video capabilities, the major development in understanding deep-sea midwater biology has been to equip submersibles with specialized samplers that collect fragile organisms and the water surrounding them undisturbed (Youngbluth, 1984).

These collection methods paint a dramatically different picture of planktonic diversity from typical net tows. Fragile lobate ctenophores, long physonects, large medusae, and species that live just above the bottom are among the groups which are exclusively available to submersibles. Submersible- or hand-collected specimens can be described in their natural state, not just based on a few fragments. A mini-resurgence of ctenophore descriptions began in the late 1970s with the first

reports of submersible-caught specimens (Madin & Harbison, 1978a, b), and this has continued to date (Harbison et al., 2001). (Interestingly, the first ctenophore described during this time appears to have been originally depicted as a juvenile lobate by Chun in 1880.) Presently, there are known to be at least two dozen new species of ctenophores, mostly in new genera, which are awaiting official description (Fig. 2; Harbison, Mills, Haddock, Matsumoto, Lindsay, personal communication; Mills, 1998). Similar situations occur in the siphonophores, although the recent efforts of Phil Pugh have gone a long way toward clarifying this group. There is one widely known species of *Apolemia,* yet at least a dozen other species are being worked up. These descriptions will have an impact on diversity similar to what occurred a hundred years ago.

Another advantage of *in situ* observations by both SCUBA and submersible is the ability to see the natural interactions between organisms in their environment. As Richard Harbison is fond of stating, sampling with plankton nets is akin to flying over London with a grappling hook. You might pick up hats and umbrellas and a few tree branches, but you can only speculate as to where hats belong, and what umbrellas are good for. Many types of predator-prey (notably gelativorous jellies) and symbiont-host relationships (notably parasitic hyperiid amphipods) have been revealed by direct observations, and by collection of specimens with their associated hosts (Harbison et al., 1977; Purcell, 1991; Lindsay et al., 2001; Raskoff, 2002; Gasca & Haddock, 2004).

Finally, these collection techniques provide access to specimens which can be used in natural experiments, and not just poorly enumerated. Specimens typically arrive at the surface alive, with their bioluminescent ability, gut contents, brooded young, and behavioral responses intact. This is essential to any study of their natural history, physiology, and ecology, and is perhaps the most significant impact of modern collection techniques.

Molecular (and other) tools

Advances in laboratory methodology have opened up areas of research that have not been possible for gelatinous creatures. At their most powerful, they convey the ability to get good information even from damaged specimens. One such technique is to perform enzyme assays to determine aerobic and anaerobic respiratory potential from tissue samples (Thuesen & Childress, 1994; Seibel et al., 1997). Analyses of stable isotope from isolated tissues can also place an organism in an approximate trophic position, although the resultant picture is sometimes softly focused. Of course the truly powerful tools are those of molecular biology; examinations of DNA, RNA, and protein provide views of the present and past lives of organisms that have never before been available.

Molecular phylogenies give additional ways to clarify relationships within phyla, but they can also allow comparisons between organisms which have few or no morphological traits in common. These analyses have revealed severely polyphyletic taxonomic groups within ctenophores and cnidarians, and have provided much of what we now know about the deep divergences in phylogenetic trees – origins which have vexed biologists for the past 150 years (Podar et al., 2001; Collins, 2002).

Molecular tools have also proven to be the keys to discovering and characterizing new fluorescent proteins and photoproteins from species which cannot be collected in large numbers. Presently it is quite possible to clone a gene from a piece of a single specimen, rather than the thousands which were required for protein purification, and this ability broadens the horizon for discovery of these and other novel proteins.

Public interest

Research takes place within the larger context of human society, and as with any research, one must consider potential benefits that justify support using public funds. Fortunately, the beauty and mystery of these organisms has sparked broad interest, and the importance of gelata is appreciated now perhaps more than ever before. Their effect on the health of the ocean has been demonstrated by the devastating impact that invasive species have had on local fisheries (Vinogradov, 1989). There has also been a suggestion that planktonic cnidarians in particular will move into the higher trophic levels which are forcibly vacated through fishing pressure (Mills, 2001). Not only will medusae thrive on the newly available prey, but they will suppress recovery of fish populations

by consuming their larvae. In addition to their danger, beauty, and effects on fisheries, jellies have provided fluorescent proteins (GFP) and photoproteins (aequorin), which are now standard and indispensable molecular and biomedical tools. Few would have anticipated the significant benefits that have come about through basic research on cnidarians.

In terrestrial systems, one must travel to remote lands to find a new species of vertebrate, yet 100 km offshore of New York, Sydney, Tokyo, or Lisbon is biologically uncharted territory, with entire families of gelata yet to be discovered. The diversity gap (the difference between known and extant species) appears to be greatest in the deep-sea and microbial realms. Thus any complete survey of diversity requires appropriate sampling of fragile deep-sea fauna.

Conclusions

Although we appear to be in an ideal time for dramatically expanding our understanding of gelata, problems still remain. Foremost among these is the lack of respect and support for the taxonomic arts. We cannot assess the impacts of environmental change (natural or anthropogenic) without narrowing the biodiversity gap through surveys of deep and formerly unattainable biota. In addition, without proper taxonomy, we cannot create robust phylogenies, and ecological studies have little validity beyond 'species X eats species Y'. An additional impediment is that the appropriate collection methods (ROVs and submersibles) are not widely available to the scientific community, and funding to explore the midwater is minimal. However, those with access to the vehicles are almost always willing to share specimens of the various phyla with other scientists. None of these problems are insurmountable; in fact, there is one overriding reason why another golden age seems inevitable: the global community of scientists devoting their immense talents to research on these fascinating organisms.

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